

MYScope
MICROSCOPY TRAINING

Transmission Electron Microscopy

Train for advanced research

Welcome

MyScope was developed by Microscopy Australia to provide an online learning environment for those who want to learn about microscopy. The platform provides insights into the fundamental science behind different microscopes, explores what can and cannot be measured by different systems and provides a realistic operating experience on high end microscopes.

We sincerely hope you find our website: www.myscope.training an enjoyable environment. In there you can explore the microscopy space and leave ready to undertake your own exciting experiments.

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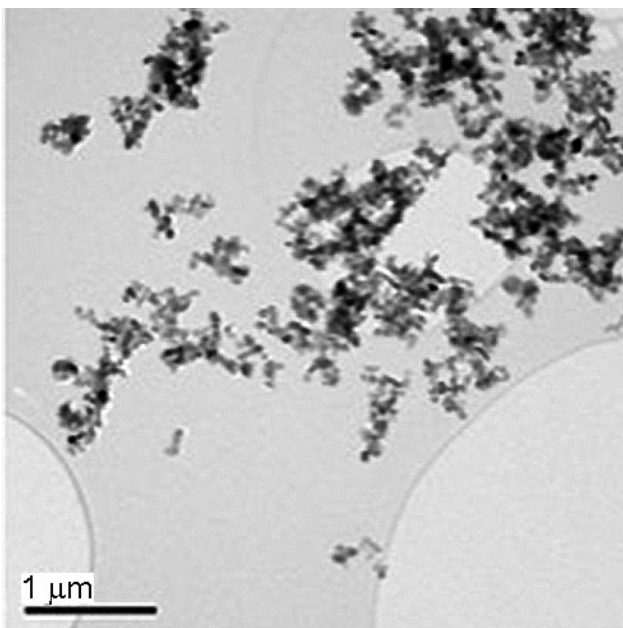
Electron energy loss spectroscopy (EELS)

Credits

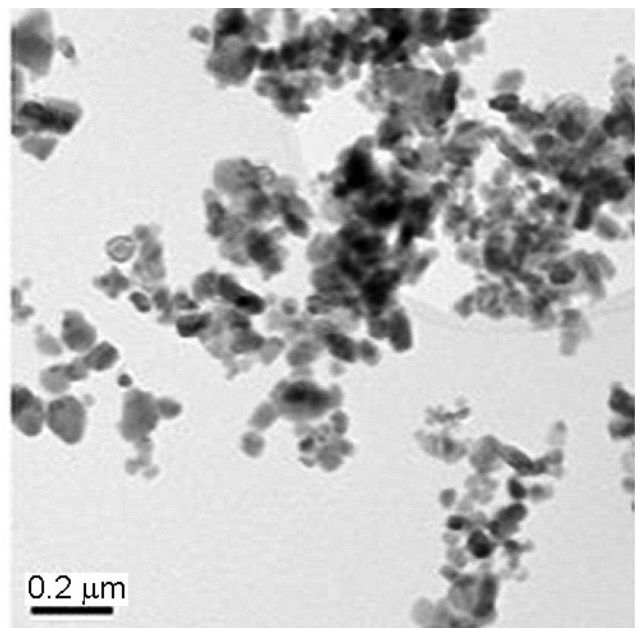
What is TEM? - Introduction to TEM

Transmission electron microscopy (TEM) is used to produce images from a sample by illuminating it with an electron beam in a high vacuum. The electrons that are transmitted through the sample are detected to form an image. Ultimately, by using a TEM we can see the columns of atoms present in crystalline samples and the molecular machinery inside cells. It allows visualisation and analysis of specimens in the realms of microspace (1 micrometre = $1\mu\text{m} = 10^{-6}\text{m}$) to nanospace (1 nanometre = $1\text{nm} = 10^{-9}\text{m}$). It is worth noting that microscopists often use the word 'micron', which means the same as micrometre.

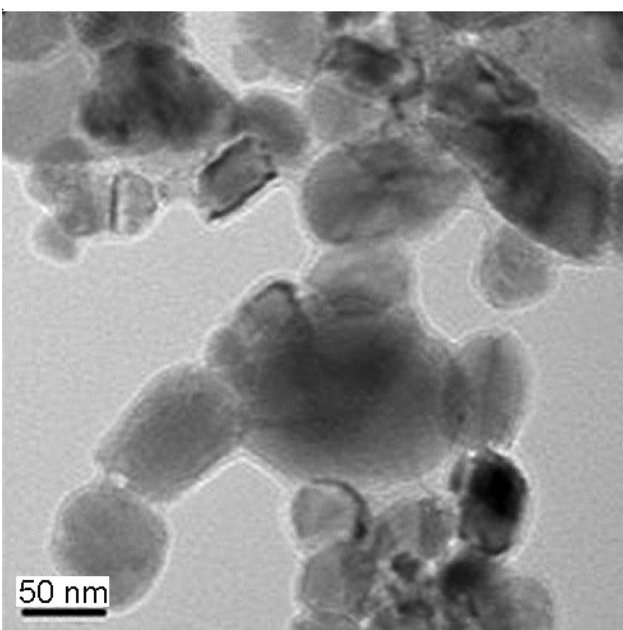
The TEM reveals levels of detail and complexity inaccessible by light microscopy because it uses a focused beam of high energy electrons. It allows detailed micro-structural examination through high resolution and high magnification imaging. It enables the investigation of tissues, cells, sub-cellular structures, proteins, crystalline and composite materials. It is also able to provide information on the orientations of crystalline phases and elemental compositions of samples containing all but the lightest elements.



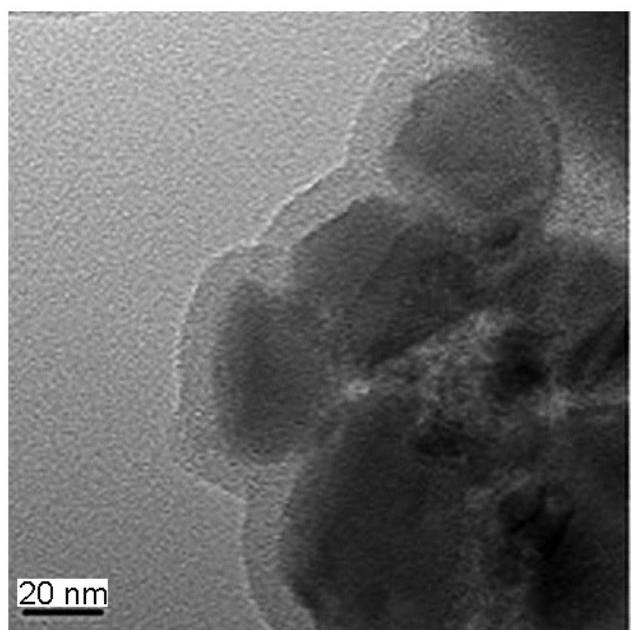
Low magnification



Medium magnification



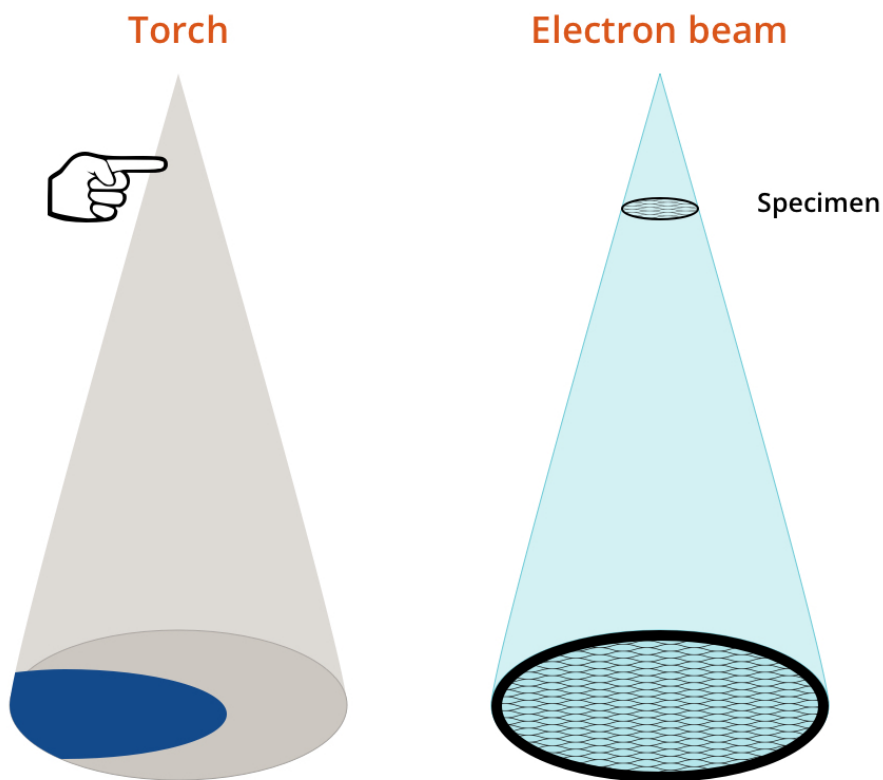
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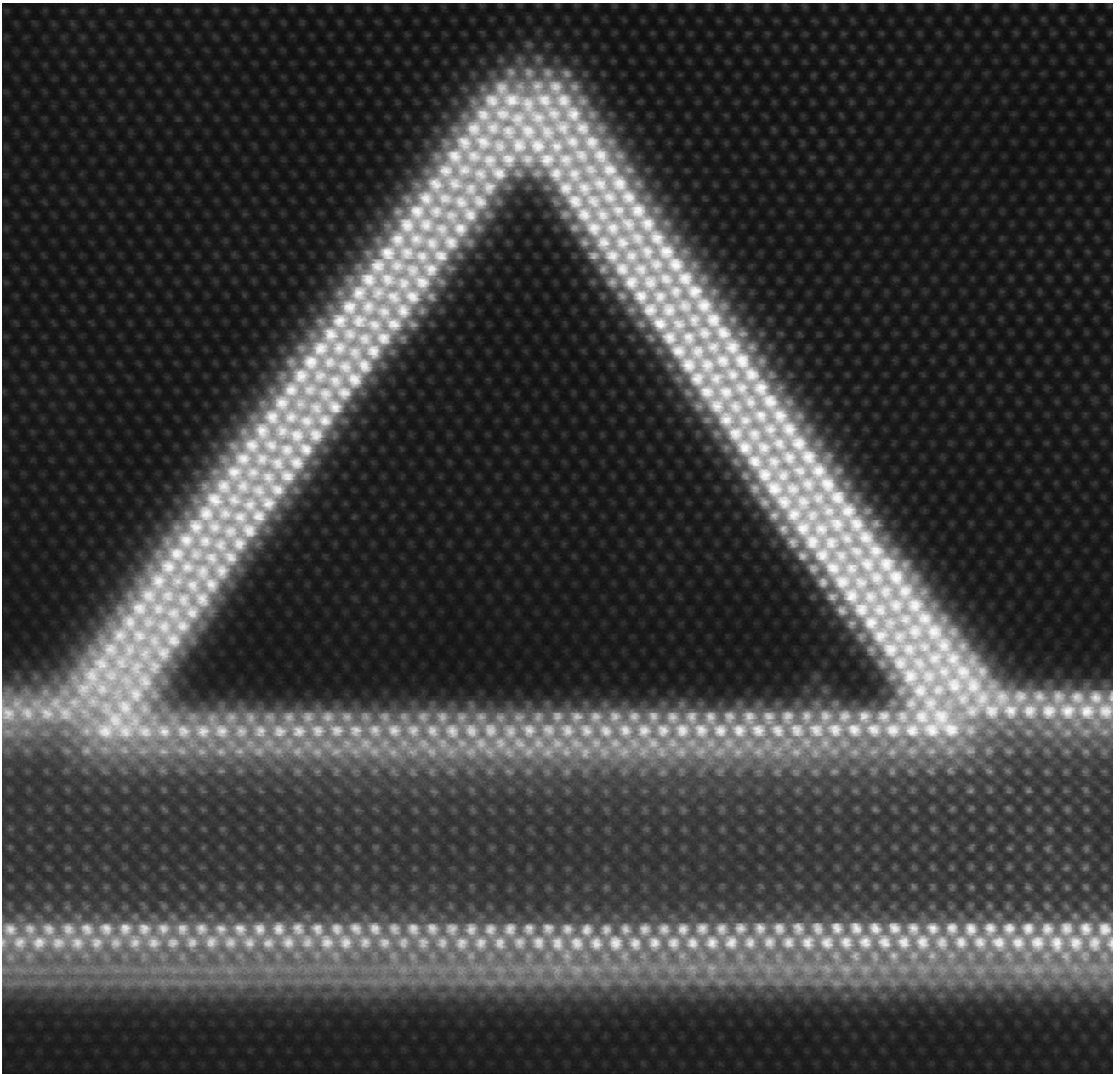
Very high magnification

Bright field TEM micrographs of the same specimen showing how different magnifications can reveal different specimen information. Low magnification images show the size and shape of the particles, whilst the higher magnification image reveals that

The word “transmission” means “to pass through”. Essentially, the way the transmission electron microscope creates a conventional image (usually termed a bright field image) of a sample can be compared to shadow puppetry. Imagine a torch beam shone through a lattice on a window. The light passes through the transparent parts of the window, but is stopped by the lattice bars. On a wall beyond, we see the lattice bars as shadows. The TEM uses a beam of highly energetic electrons instead of light from a torch. On the way through the sample some parts of the material stop or deflect electrons more than other parts. The electrons are collected from below the sample onto a phosphorescent screen or through a camera. In the regions where electrons do not pass through the sample the image is dark. Where electrons are unscattered, the image is brighter, and there are a range of greys in between, depending on the way the electrons interact with, and are scattered by, the sample.



Magnifications of up to 1,000,000x and resolution below 1 nm are achieved routinely on the best instruments. A scale bar is essential on a TEM image to allow the actual size of structures in the image to be calculated.

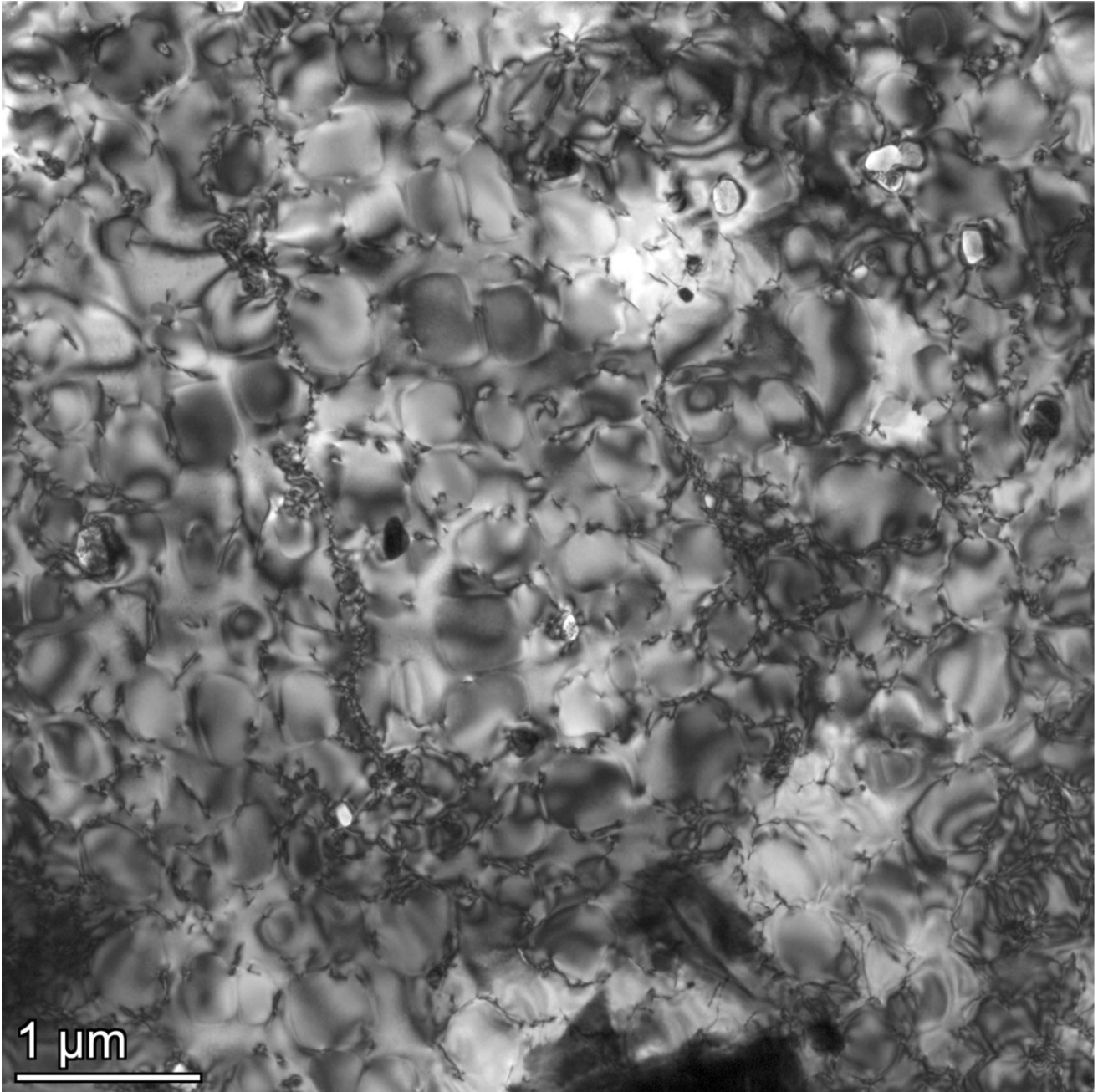


An example of HAADF STEM imaging of a precipitate in an Al-Cu-Ag alloy. The precipitate appears brighter due to atomic number contrast. Image courtesy Julian Rosalie and Laure Bourgeois, Monash Centre for Electron Microscopy.

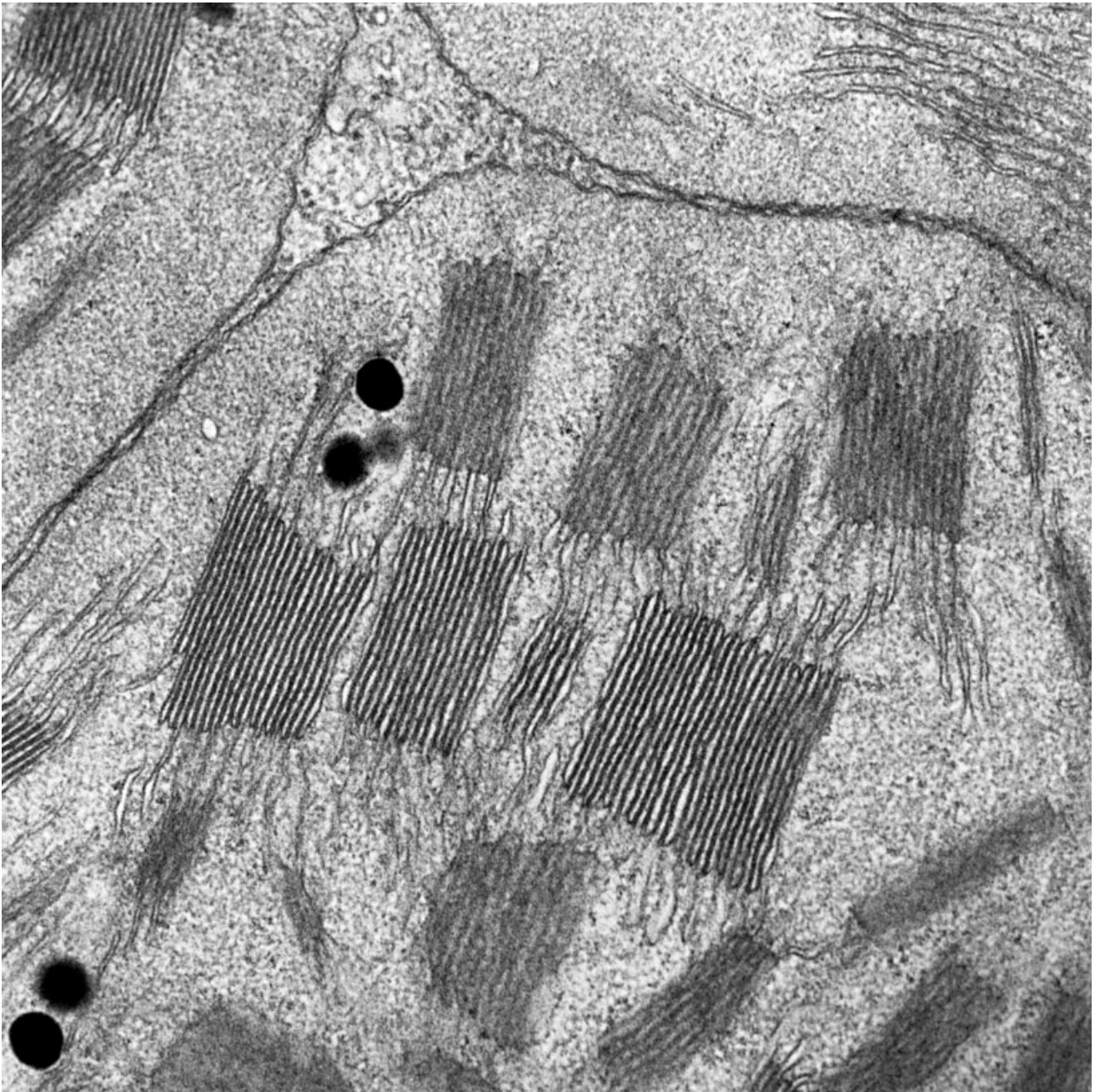
Key advantages

The TEM provides the user with advantages over the light microscope in three key areas:

1. Resolution at high magnification. Resolution can be defined as the smallest distance between two closely opposed points, at which they may be recognised as two separate entities. The best resolution possible in a light microscope is about 200 nm whereas a typical TEM has a resolution of better than 1 nm. This enables visualisation of features in crystal lattices, such as defects and dislocations, as well as sub-cellular structures such as organelles and molecular machinery.

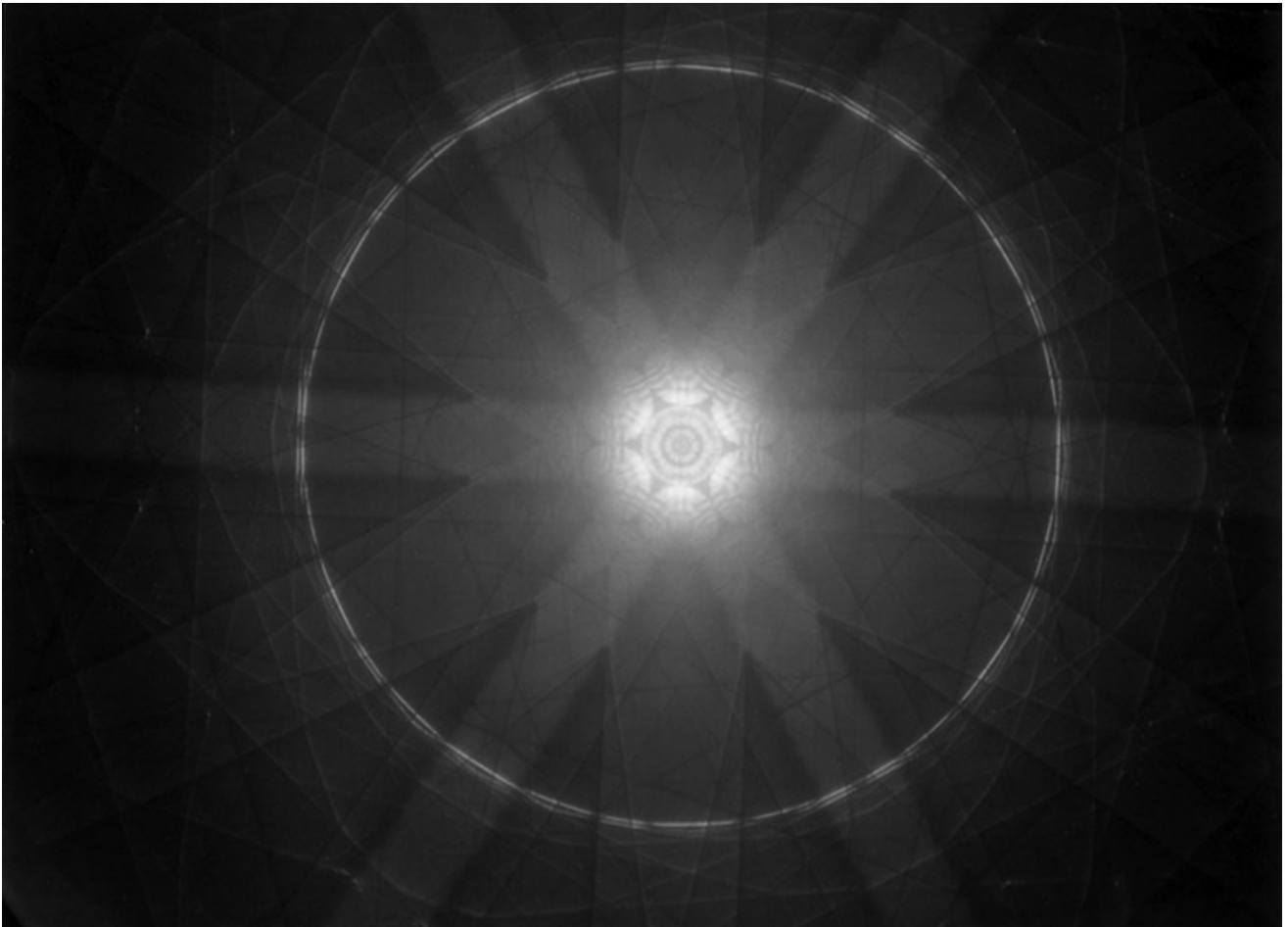


Dislocations in an additively manufactured Ni-based superalloy. Credit: Zibin Chen and Bryan Lim, University of Sydney.

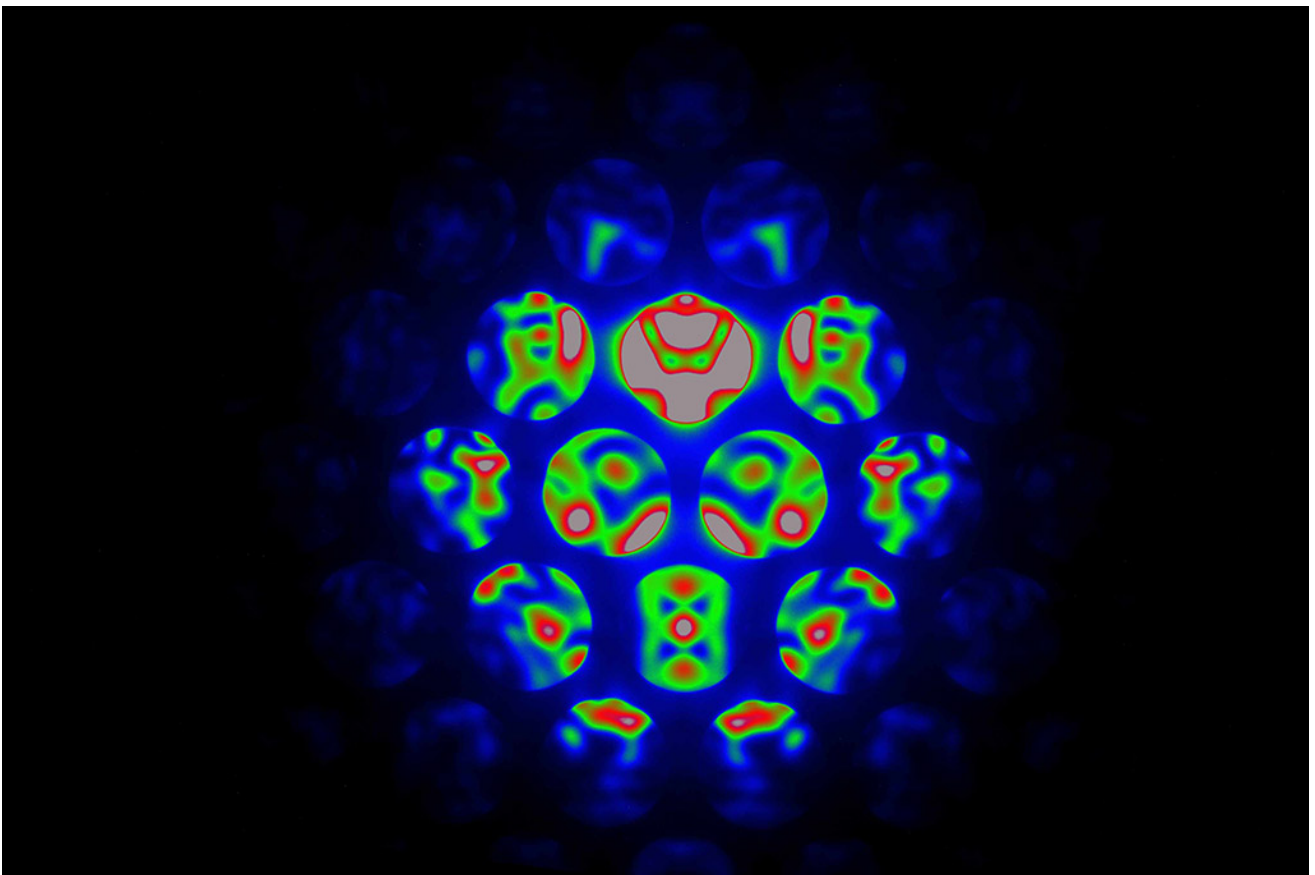


Structures within a chloroplast. Credit: Ian Kaplin, University of Sydney.

2. Structural information through diffraction. If the material being viewed has a periodic structure, like that in a crystal, then the beam can interact with that structure in such a way that it diffracts. The ability to form diffraction patterns of this kind is unique to the TEM, and provides information on crystal structure, symmetry and orientation of the crystal being viewed.

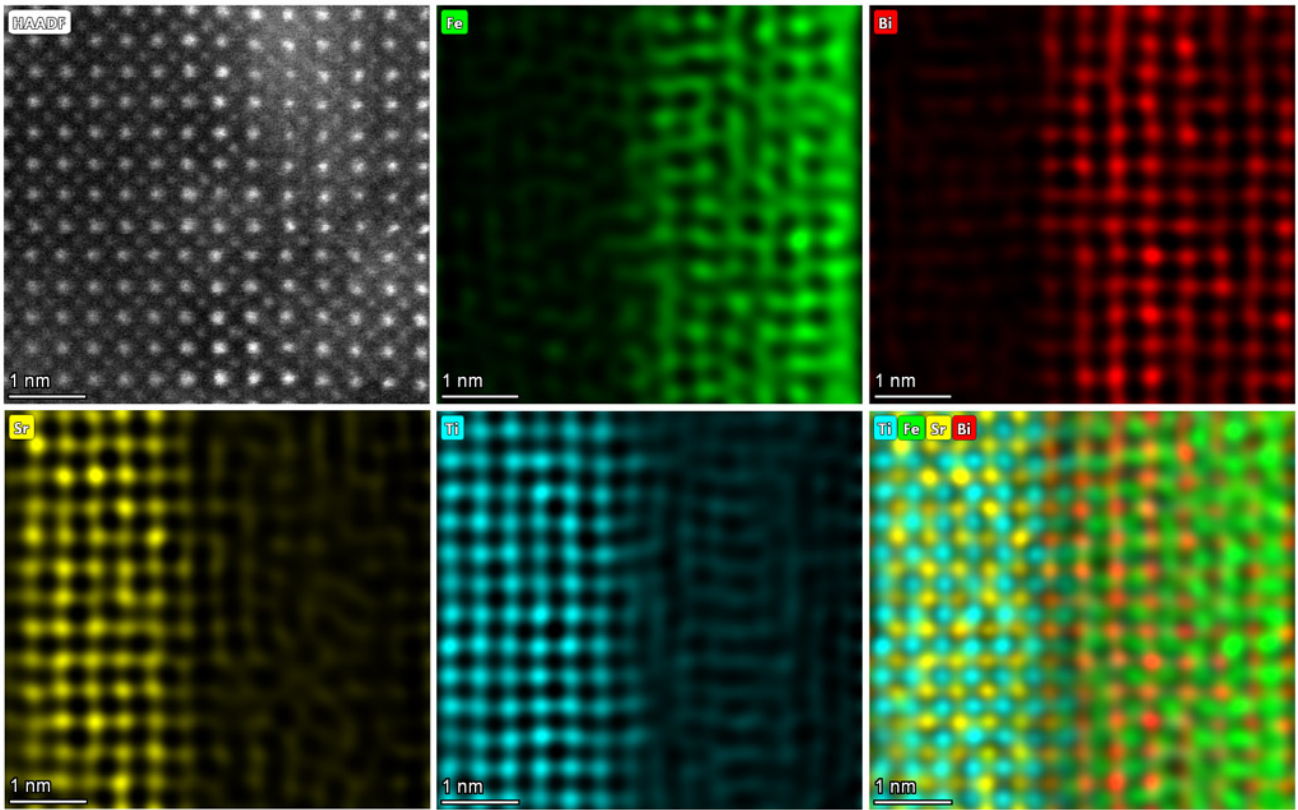


Diffraction pattern from crystalline specimen taken on a major zone axis. Image courtesy of Roger Wepf, University of Queensland.



Quantitative convergent beam electron diffraction pattern. Image courtesy Andy Johnson, University of Western Australia.

3. Microanalysis. Analysis of sample elemental composition can be performed in the TEM. This is also possible in the SEM, but not at the high resolutions possible in TEM.



Energy dispersive spectroscopy imaging of the interface between BiFeO_3 and SrTiO_3 . Image courtesy of Jiangtao Qu, University of Sydney.

What the TEM can do

The main advantage of the TEM is the ability to reach high magnifications and these can be used to examine the structure, composition, and properties of specimens in great detail. TEM is used in many fields such as: biological and medical science, materials science, geology, environmental science, nano-technology and protein studies, among others. It is used to study the morphology, structure, and local chemistry of sub-cellular components, viruses, metals, ceramics, and minerals. It can be used to study crystal structures, orientations and chemical compositions of phases, precipitates and contaminants through diffraction patterns, characteristic X-ray, and electron energy loss analysis. For further information on specialist techniques, please refer to the Specialist Techniques section at the end of this module.

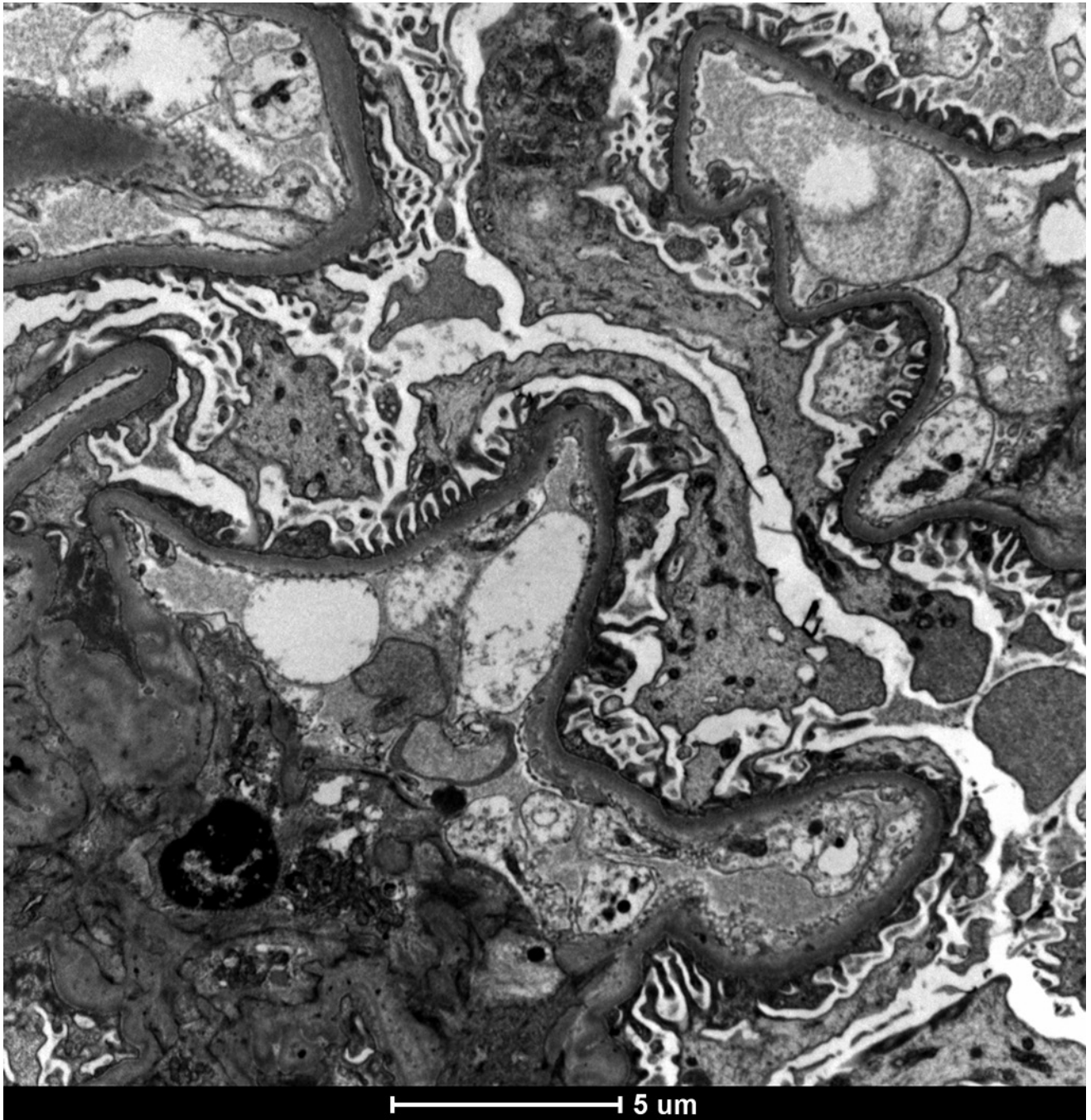


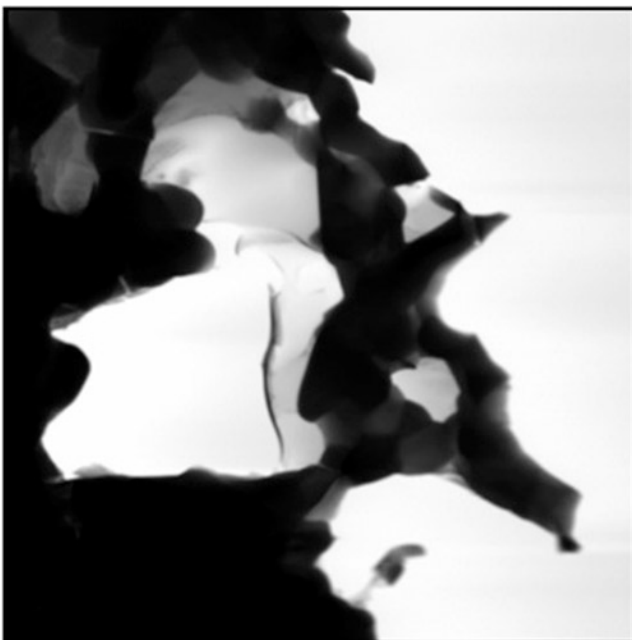
Image courtesy Ruth Williams, Adelaide Microscopy.

What the TEM can't do

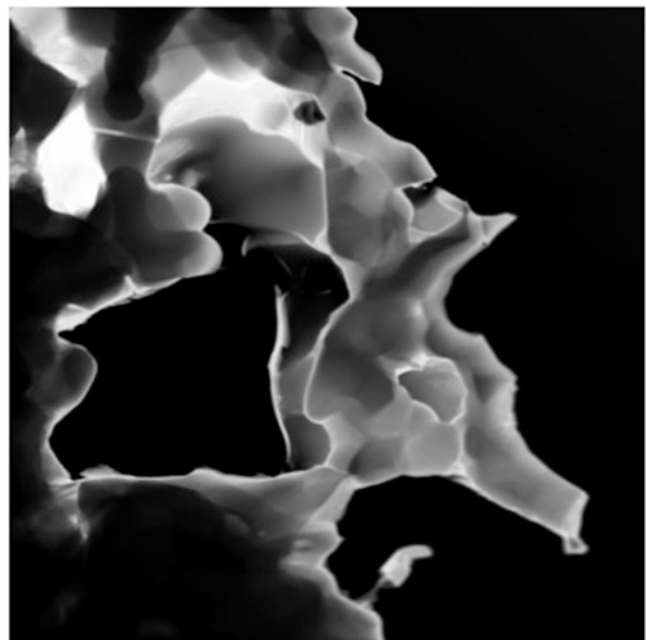
There are some things TEM can't do:

- TEM cannot take colour images. Colour is sometimes added artificially to TEM images after they have been captured.
- TEM cannot image through thick samples: the usual sample thickness is around 100-200nm. Electrons cannot readily penetrate sections much thicker than 200nm.
- A standard TEM cannot image surface features.
- The TEM cannot reliably image charged molecules that are mobile in a matrix. For example, some species (e.g. Na⁺) are volatile under the electron beam because the negative electron beam exerts a force on charged material. Many specimens require specialist sample preparation, and some biological specimens require staining with a heavy element to reveal the features of interest. Without these specialist preparation techniques, TEM observation will not be successful.

Where the TEM is not suitable, other techniques should be used. If in doubt consult the [Technique Finder](#) on the Microscopy Australia web site.



Bright field

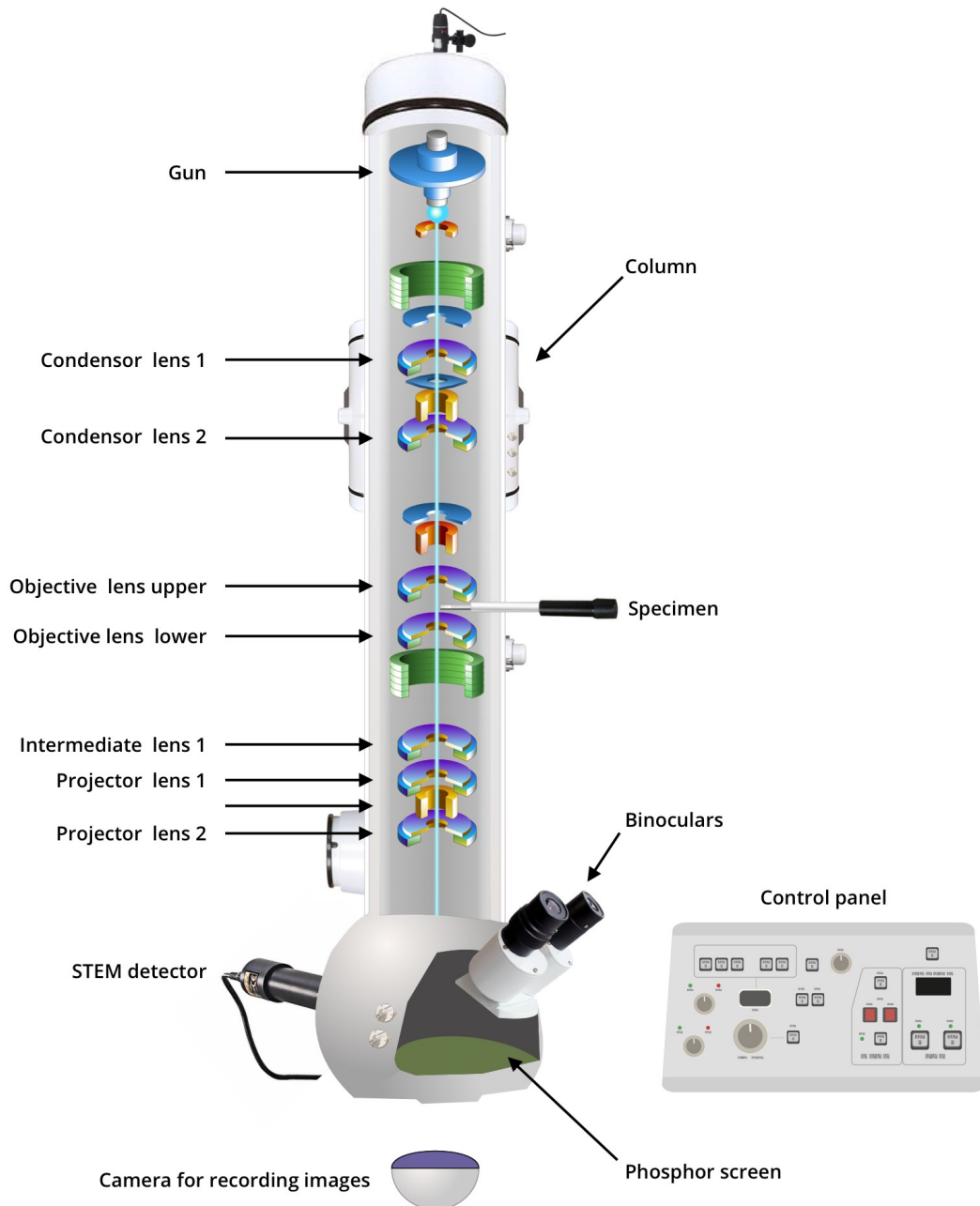


Dark field

Example of bright field and dark field imaging in the TEM. Image courtesy of Roger Wepf, University of Queensland.

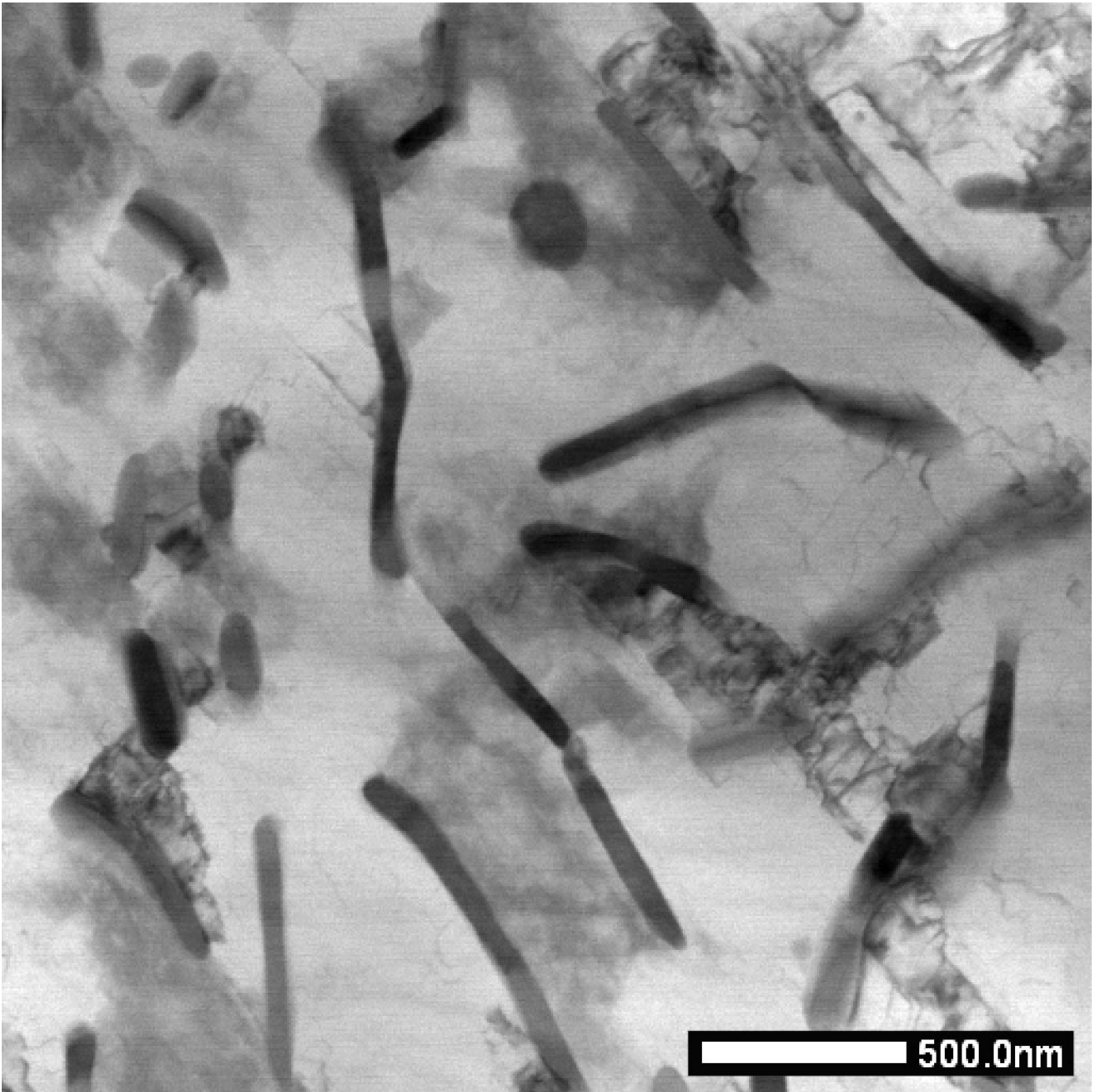
How does a TEM work? - Overview of TEM workings - Instrument design

The components of a TEM are arranged in a long column with the electron-producing gun at the top. The electron beam generated by the electron gun passes down the column through a series of electromagnetic lenses, through the sample to an image collector at the bottom.



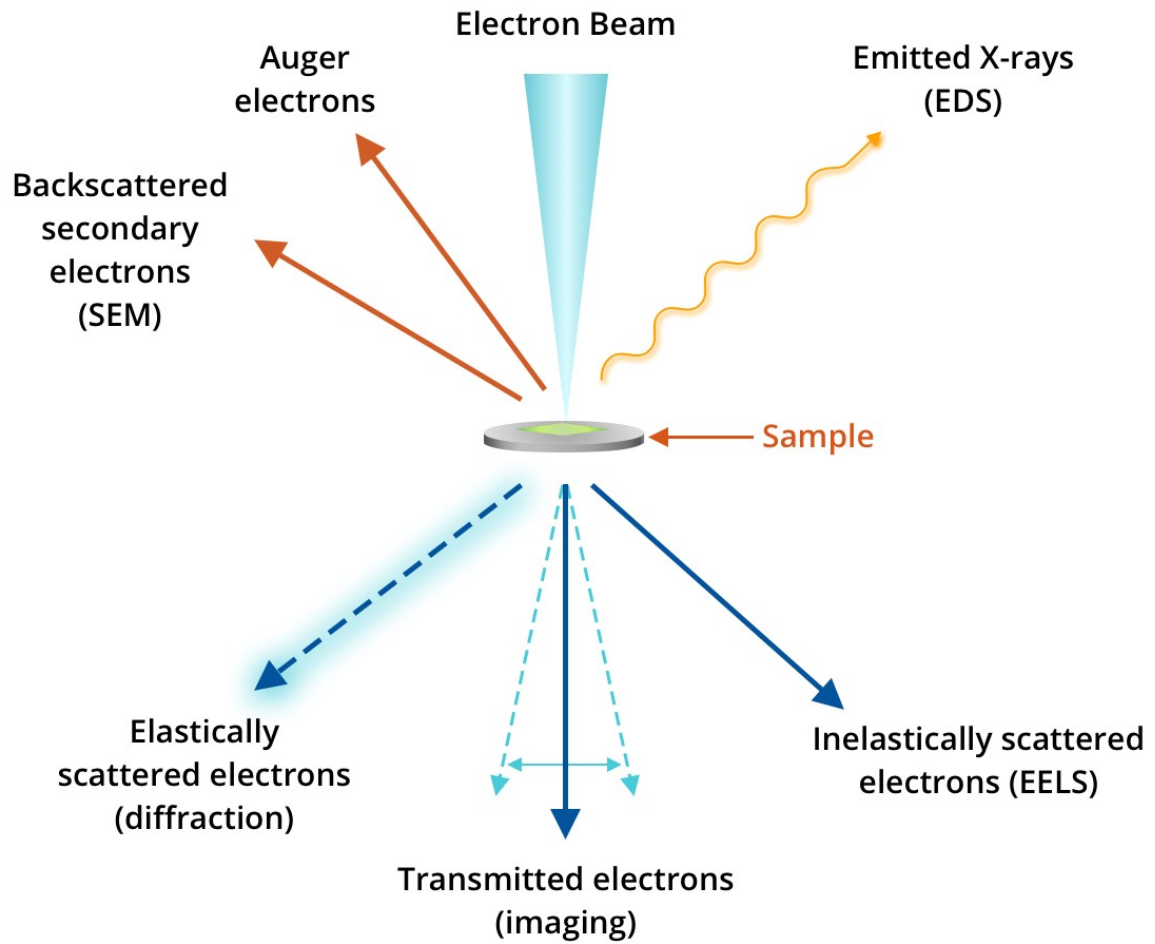
Schematic illustration of a TEM showing some components of interest.

On the way through the sample some parts of the material stop or deflect electrons more than other parts. The electrons are collected from below the sample onto a phosphorescent screen or through a camera. In the regions where electrons do not pass through the sample the image is dark. Where electrons are unscattered, the image is brighter, and there are a range of greys in between depending on the way the electrons interact with and are scattered by the sample.



There are many interactions between the beam and the specimen and some of these are shown in the diagram below. These are discussed in the SEM module.

Electron interactions with the specimen



Resolution

The concepts of resolution and magnification are often confused. These and many other basic concepts are covered in the Microscopy Basics module.

Illumination with a smaller wavelength beam results in better resolution (the two spots can be seen as distinct) and this is why the electron microscope produces higher resolution images than the light microscope; because the wavelength of an electron is smaller than that of visible light. Resolution in a microscope is determined primarily by the wave nature of the beam according to Abbe's equation:

TEM resolution equation

$$d = \frac{0.61\lambda}{n \sin \alpha}$$

Where d = resolution (minimum resolvable distance)
 λ = wavelength of energy source
 n = refractive index of the medium
 α = aperture angle

The wavelength of an electron is dependent upon accelerating voltage and is given by:

TEM wavelength equation

$$\lambda = \frac{h}{\sqrt{2meV}}$$

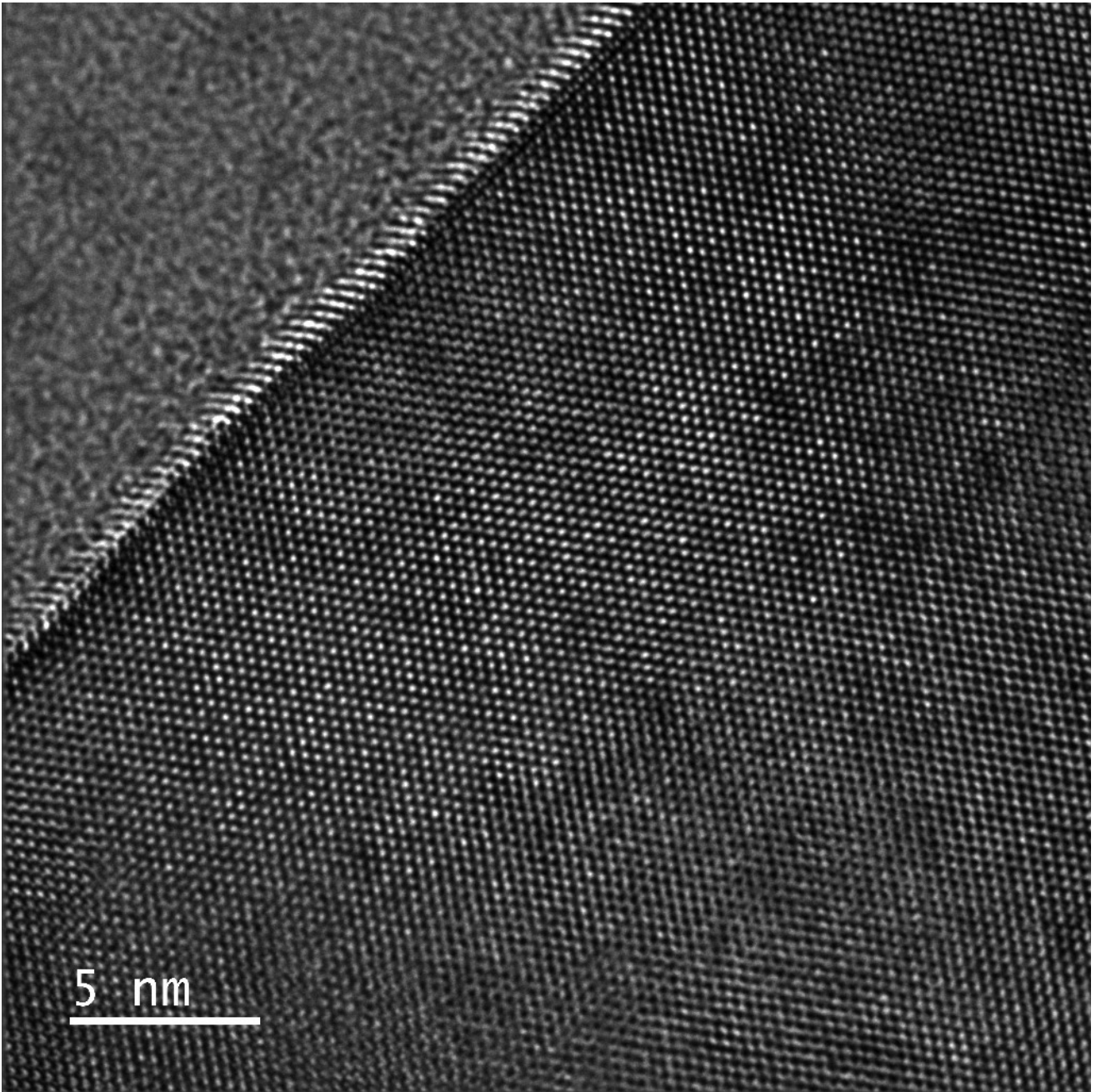
Where h = Planck's constant (6.626×10^{-34} j s)
 m = electron mass (9.109×10^{-31} kg)
 e = electronic charge (1.60×10^{-19} C)
 V = accelerating voltage ($0.5 - 3 \times 10^4$ V)

This equation can be approximated to:

$$\lambda = \sqrt{\frac{1.5}{V}} \text{ nm}$$

| Voltage (kV) of | Becomes | λ (nm) |
|-----------------|---------|----------------|
| 5 | → | 0.0173 |
| 10 | → | 0.0122 |
| 200 | → | 0.0127 |
| Light | → | 400 - 700 |

The higher the accelerating voltage, the smaller the wavelength of the electrons and the higher the possible achievable resolution

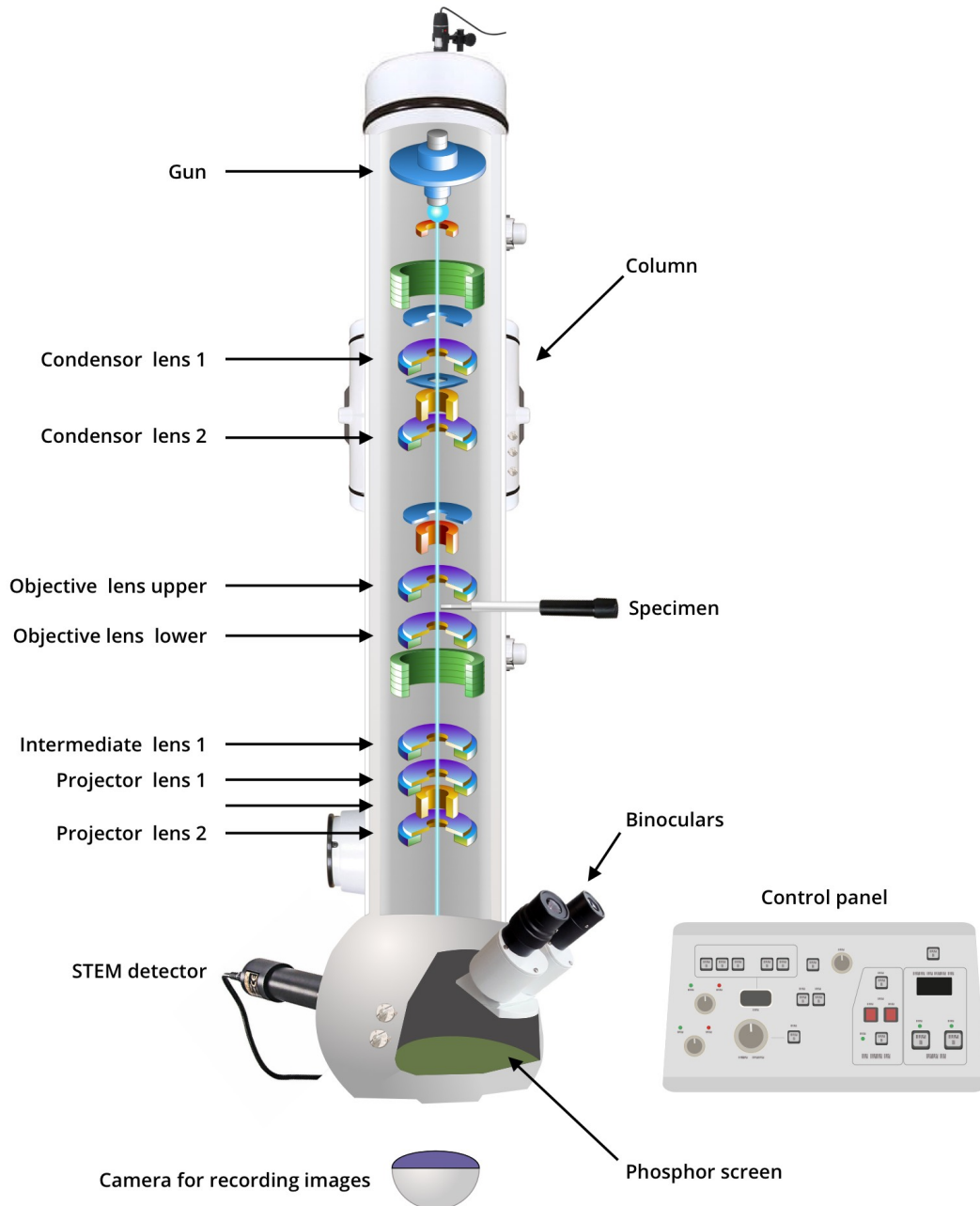


High resolution TEM of amorphous platinum coated onto ITO. Image courtesy of Jiangtao Qu, University of Sydney.

Components of a TEM - Introducing TEM components

The typical transmission electron microscope laboratory contains a machine with these components:

- Electron gun
- Electron column
- Electro-magnetic lens system
- Detectors
- Specimen/sample chamber
- Main control panel and operational controls
- Image capture system



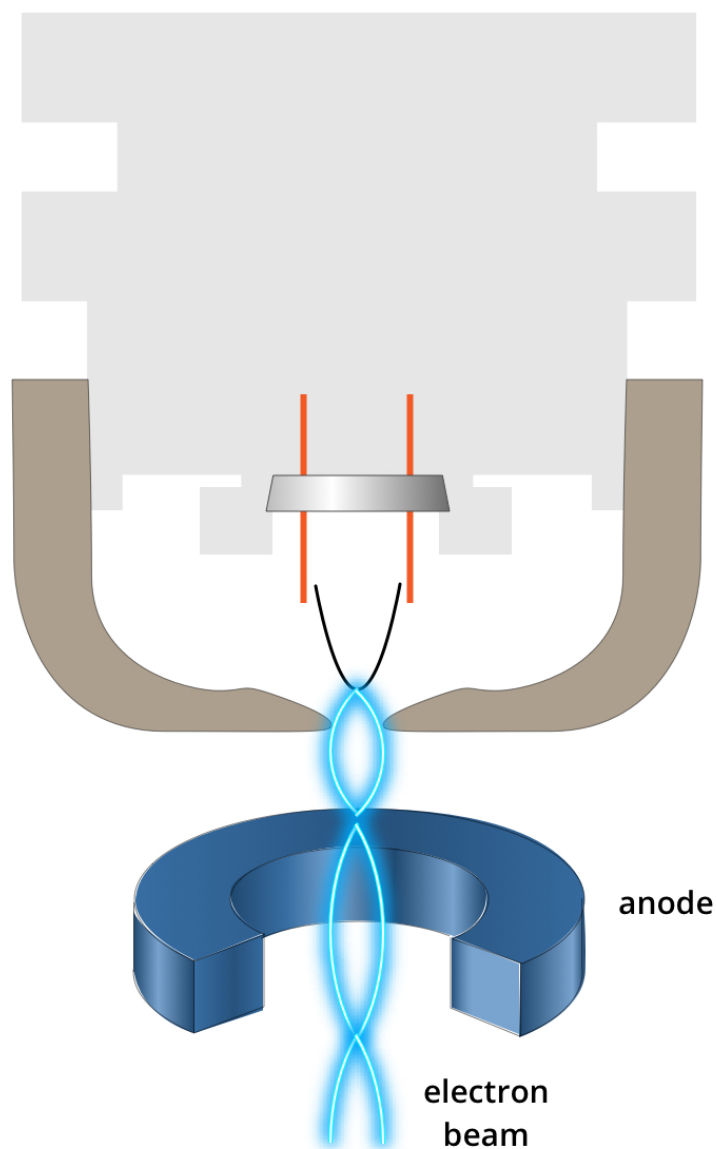
Vacuum system

The vacuum is important because the particles in air can deflect the trajectory of the electrons in the electron beam. This in turn affects the quality of the final image, decreasing both the resolution and the signal-to-noise ratio.

Electron gun

The electron gun generates the electron beam. It is usually positioned in the top of the instrument column. There are several different kinds of electron guns available, and the one shown here is the more simple design where electrons are pulled off a tungsten filament and then accelerated down the column. The kind of electron gun fitted will determine the ultimate resolution of the instrument.

Electron gun



Electron column

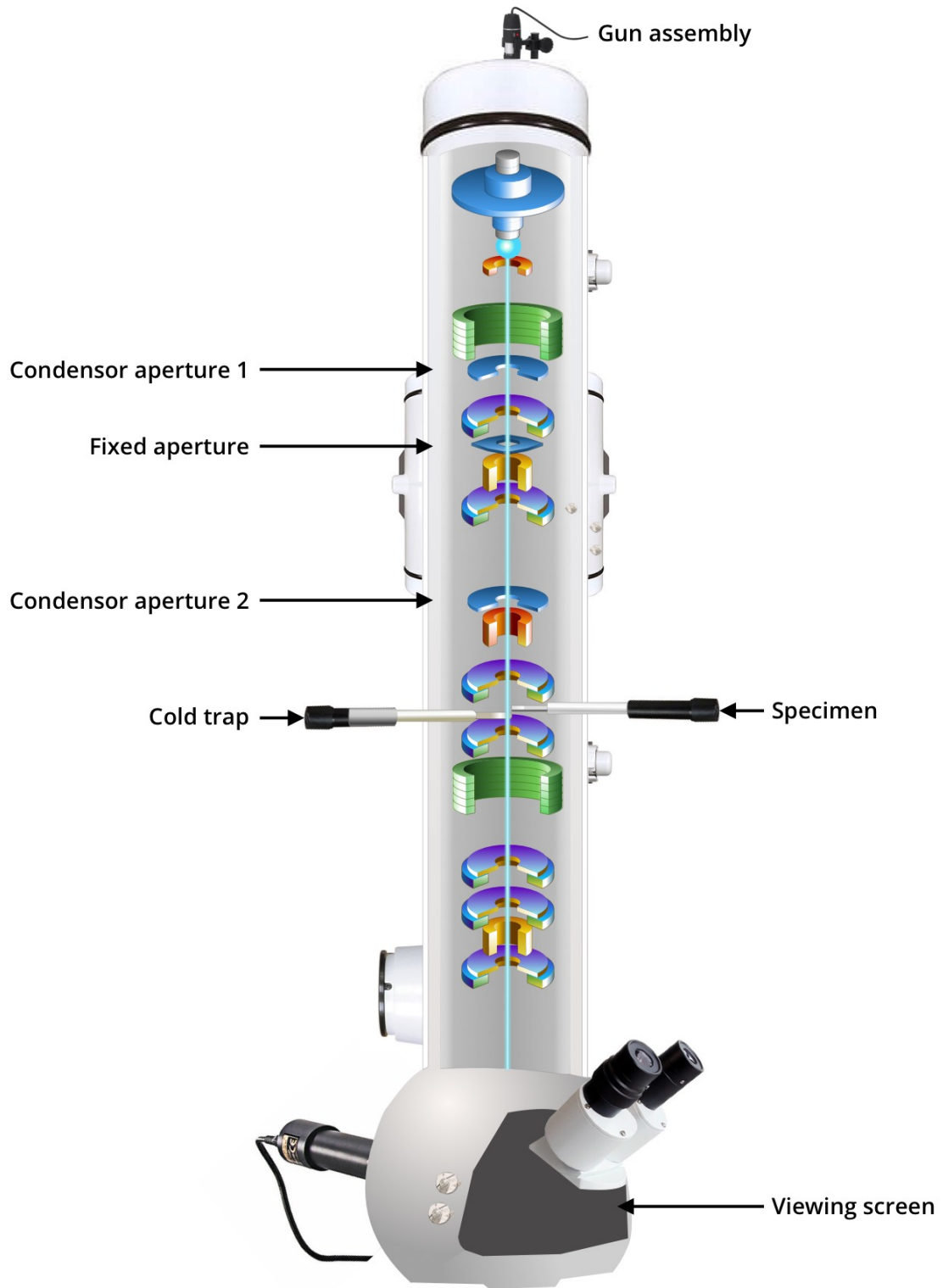
The electron column is made up of the gun assembly at the top, a column filled with a set of electromagnetic lenses, the sample port and airlock, and a set of apertures that can be moved in and out of the path of the beam. The contents of the column are under vacuum.

The apertures can be easily removed from the beam path by the user. This is important for operation of the objective and selected area diffraction apertures during imaging.

The apertures are located within aperture strips, typically consisting of a strip of molybdenum containing a sequence of different sized holes, that allow modulation of the beam to different degrees of precision.

Adjacent to the sample port is a 'cold trap'; this consists of a liquid nitrogen dewar containing a conductive metal tassel which is joined to a rod at the top (see arrow path in image). The rod penetrates the column and sits near the sample. This cold area acts as a condensation site that adsorbs material that leaves the sample. Such material can contaminate the chamber or affect the vacuum status of the machine.

At the base of the column is the sample viewing chamber. This has a screen which produces an image via fluorescence when impacted by the projected electrons. A set of binoculars is attached to the column and is used for finely focusing the image. There is also a camera that can be inserted into the beam.



Electromagnetic lenses

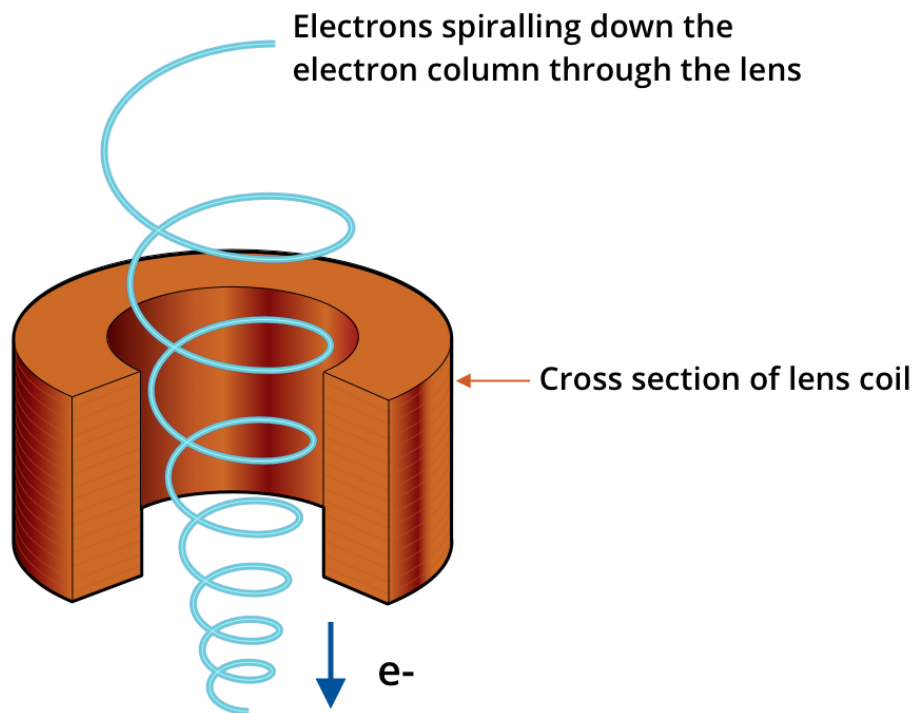
Within the column the electromagnetic lenses shape the electron beam. Since the electron beam is negatively charged, an electromagnetic force can be used as a lens. Wire coils surround the beam and produce a field that generates a deflecting force on the electrons. Each lens is constructed of a coil of copper wire through which a current runs. There is a hole in the center through which the beam travels. Magnetic lenses allow the user to change magnification and focus of the beam. A magnetic electron lens has two parts:

- a pole-piece: a cylindrically symmetrical core of soft iron with a hole drilled through it (bore)
- a coil of copper wire that surrounds each pole-piece.

When we pass a current through the coil, a magnetic field is created in the bore. The strength of the field in a magnetic lens controls the ray paths, bringing off-axis rays back to focus.

Comparing the action of an electromagnetic lens with an optical lens we see that the image is rotated, to a degree that depends on the strength of the lens. Focal length can be altered by changing the strength of the current.

The resistive heating of the coil means that the lenses have to be cooled and a water cooling system is an essential part of TEM lenses.



Specimen/sample chamber

The sample is loaded into the well in the specimen holder via a flange or a ring that screws into the well to hold it securely in place. It is imperative that the grid is secured so that it does not fall out of the specimen holder.

The holder is then inserted into the column. During this process the sample airlock is evacuated, which can take a few minutes. It is important to keep the O-ring on the holder free from lint or dust and properly greased or it can interfere with the vacuum. To keep it clean, the holder is stored in a covering sleeve when outside of the machine.

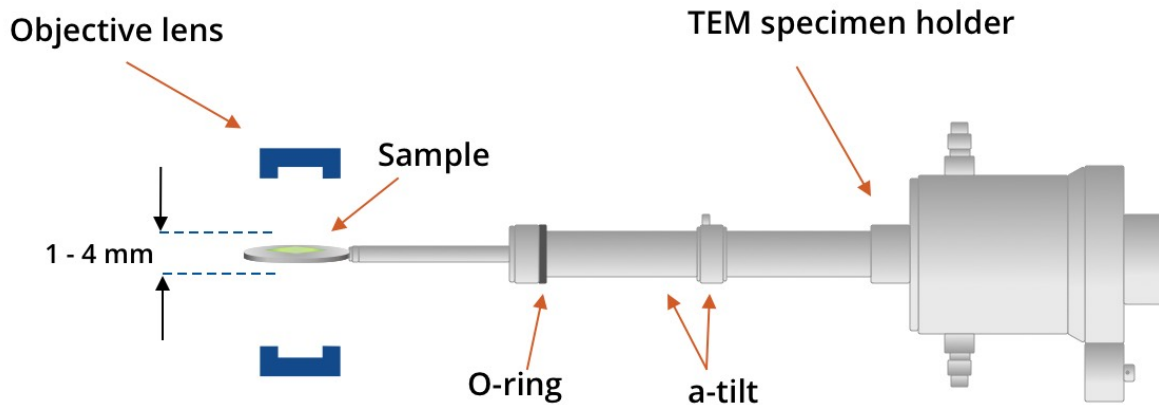
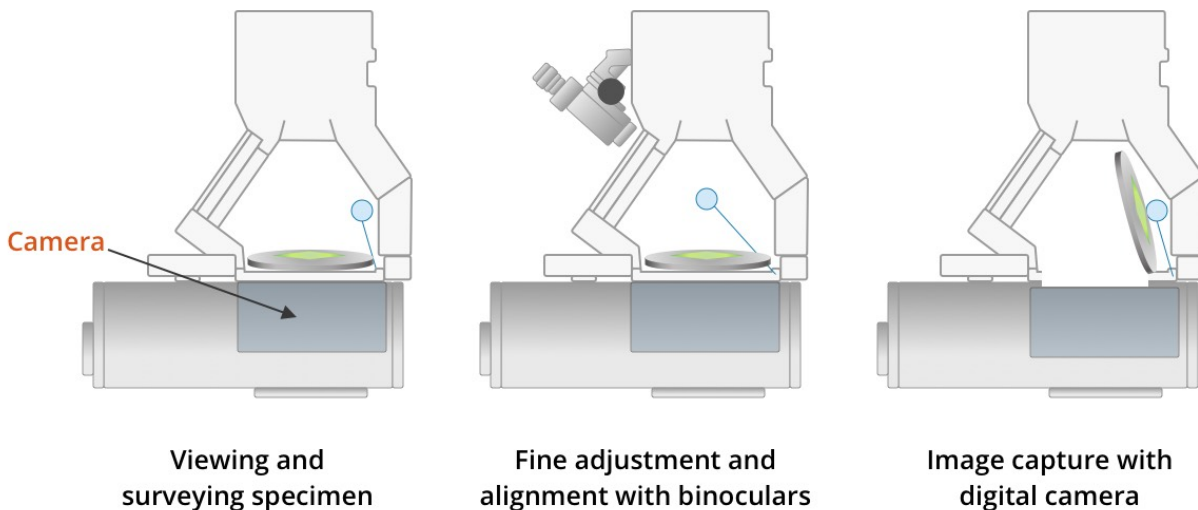


Image capture

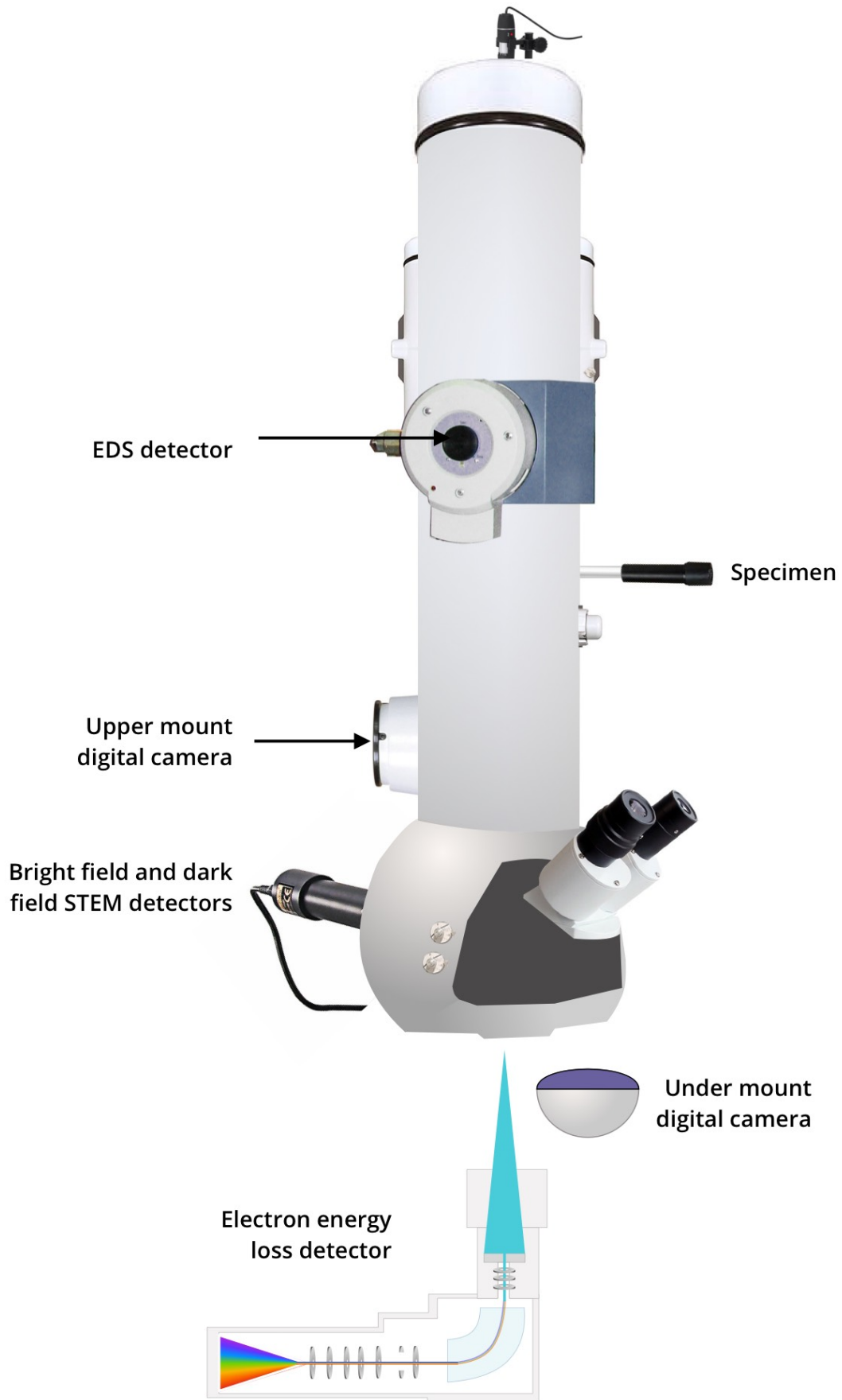
The image is projected onto the screen in the viewing chamber, and binoculars are available for focusing the image. A mini-screen is provided for use with the binoculars. The main viewing screen in the chamber is only used for beam alignment and surveying the specimen. To collect a permanent image, a camera is inserted into the path of the beam. This allows the image to be collected in a digital form. For cameras and detectors located below the viewing screen, the entire phosphor screen can be tilted out of the beam path.

The exposure time (the length of time that the beam is directed at the collection device) can be adjusted to suit beam parameters and to control the quality of the desired image.



Detectors

There are many possible detectors that can be used on a TEM. All TEMs have a phosphor viewing screen. Many years ago the image was recorded on physical film, but now we record images digitally using cameras that can be mounted either above or below the viewing screen. Diffraction work is often carried out on cameras above the viewing screen, and high resolution imaging tends to be carried out using the under-mount camera. Almost all TEMs have a facility to scan the beam across the sample, and the scanning TEM detectors are positioned just above the viewing screen. Advanced energy loss imaging can be carried out by specialised equipment mounted below the viewing screen, see the specialist techniques section for more information on this. Chemical information can also be detected using an energy dispersive spectroscopy system that works in a very similar way to the same technique in SEM. The EDS detector is positioned high in the column immediately above the specimen.

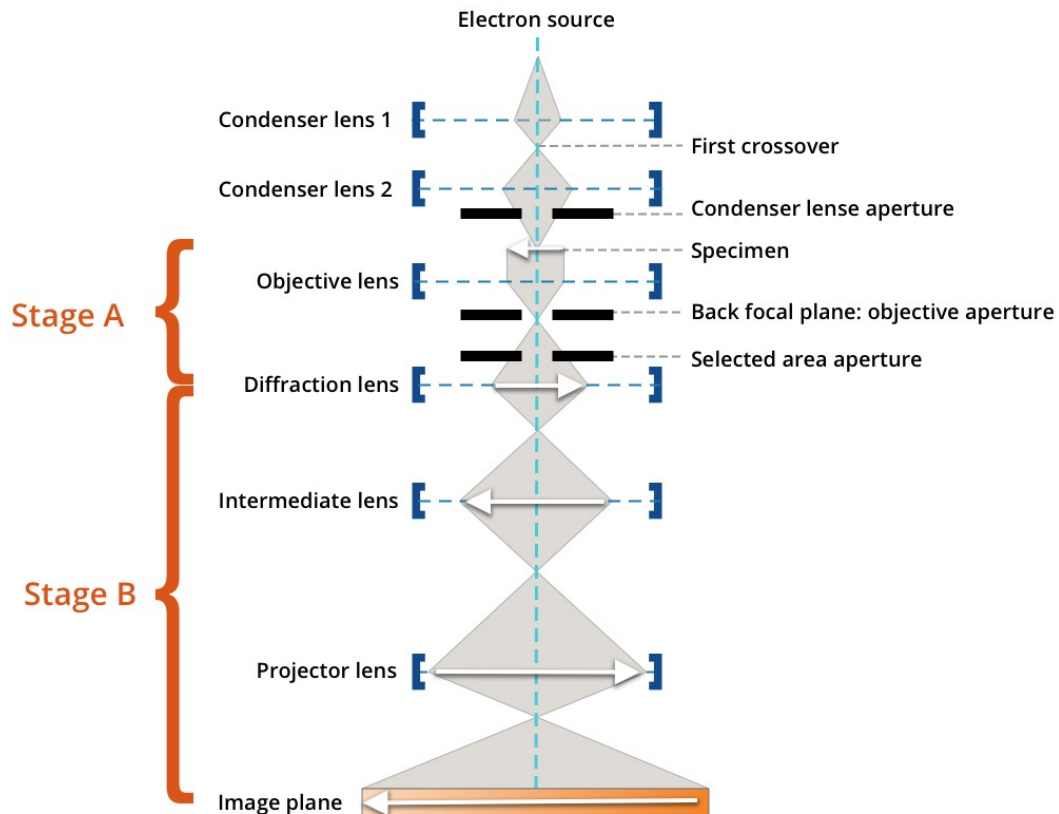


How images are formed - Image formation basics

Image formation in the TEM is complex and occurs in two stages.

Stage A is the scattering of an incident electron beam by a specimen. This scattered radiation passes through an objective lens, which focuses it to form the primary image.

Stage B uses the primary image obtained in stage A and magnifies this image using additional lenses to form a highly magnified final image.



In the process of forming the primary image, the objective lens produces a diffraction pattern at its back focal plane. The back focal plane is at the same position as the objective aperture, and people often use the edge of this aperture to focus the diffraction pattern. The planes within the TEM imaging system are summarised here:

- The object plane is where the sample sits and contains the object point. It is always located above the lens.
- The image you see is formed at the image plane. It contains the image point and is always located below the lens.
- The focal plane of the lens is where parallel rays are brought to a focus.

Object/Image planes

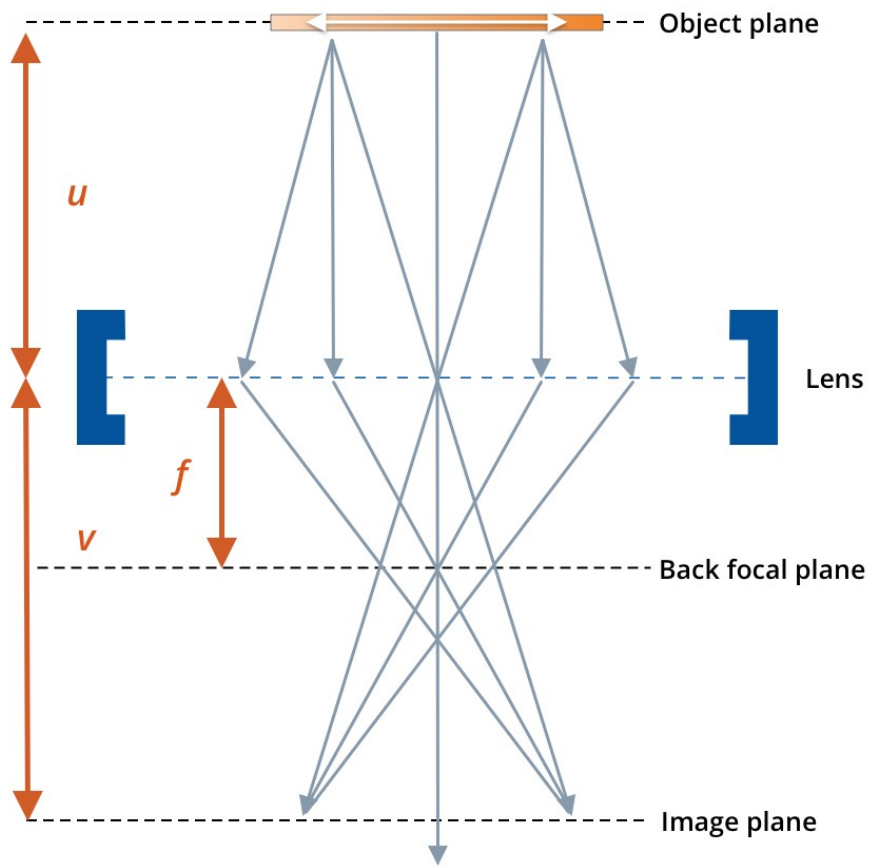
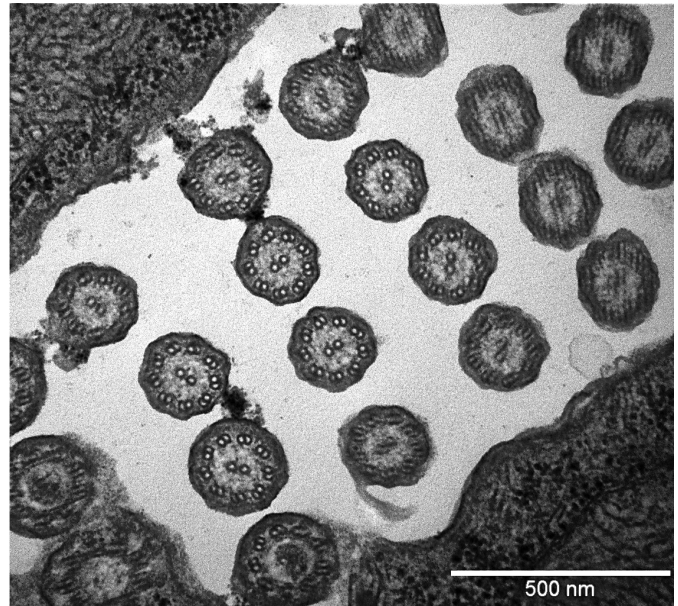
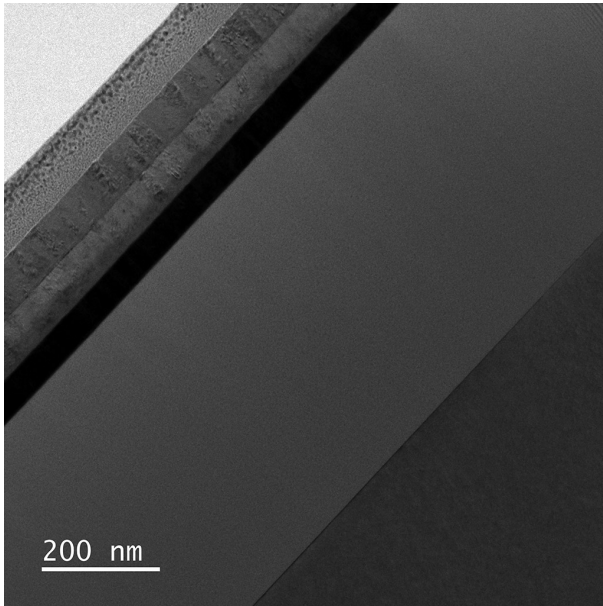


Image types - Bright-field images

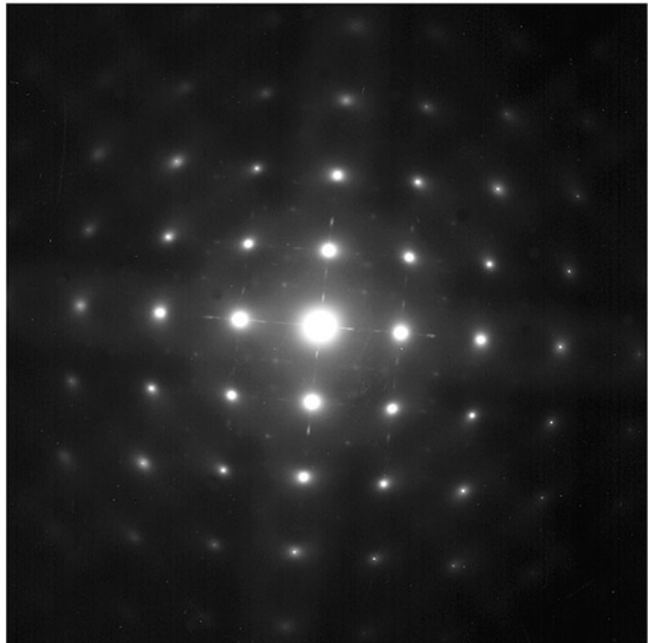
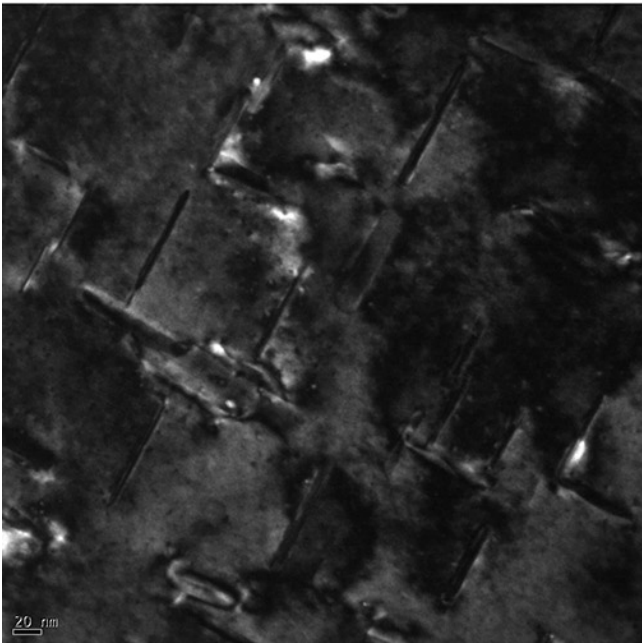
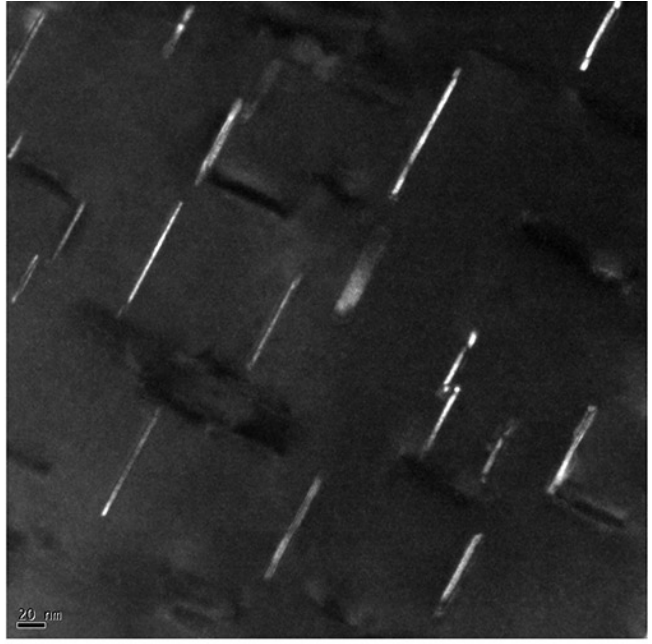
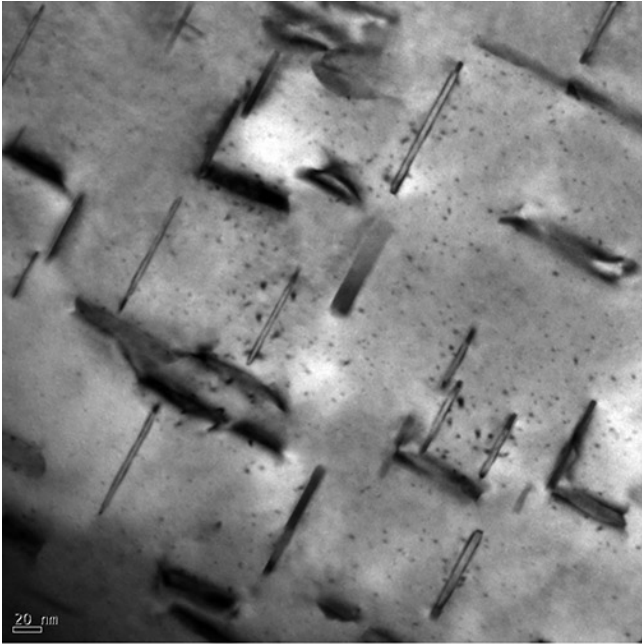
The most common type of image generated using a TEM is a bright-field image. Some areas of the sample scatter or absorb electrons and therefore appear darker. Other areas transmit electrons and appear brighter. In simple terms the bright-field image appears as a shadow of the specimen. In the bright-field image the objective aperture is used to select the unscattered electron beam. In doing so, the scattered electrons are excluded from forming the image. This aperture enhances the contrast in the image.



Left image shows a bright field TEM image of a multilayered coating on silica. Image courtesy of Jiangtao Qu, University of Sydney. Image on the right shows cilia in protozoan, image courtesy Ruth Williams, Adelaide Microscopy.

Dark-field images

Dark-field images are produced using the primary aperture to exclude the primary (unscattered) beam from the image collected below the sample. The image is produced only by the scattered electrons. Regions where no scattering occurs, such as where the primary electron beam passes straight through the sample, appear black (e.g. in areas around the sample). This kind of imaging is useful in studying crystal defects, and for the imaging of specific crystallographic phases. Other forms of dark field imaging are discussed in the section on Specialist TEM techniques later in this module.



Example of dark-field imaging in an aluminium alloy. Top left shows bright-field image, top right shows the same region in dark-field mode highlighting one variant of the precipitates. Image on bottom left shows the use of dark-field imaging to highlight local strain fields around particles. Diffraction pattern from the same region shown on the bottom right. Images courtesy of Matthew Weyland, Monash Centre for Electron microscopy.

Diffraction

Diffraction patterns occur due to elastic interference (diffraction) of the electron beam as it passes through crystalline specimens. This can be most easily understood by remembering that the electron beam has wave-like properties. The principles that govern the diffracted spot are the same as those discussed in the XRD module of MyScope. The ability of the TEM to form these diffraction patterns is unique to this kind of instrument, but does require advanced understanding of the path of the electron beam within the instrument, and also a strong understanding of crystallography. For these reasons the sections on diffraction are further discussed in the specialist techniques section at the end of this module.



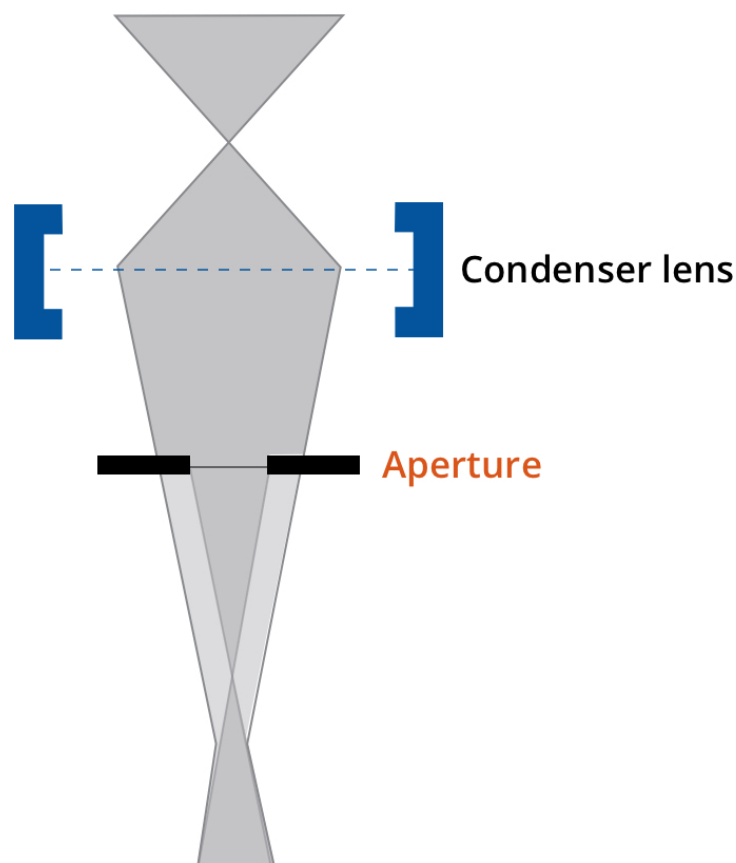
Selected area diffraction pattern of quasicrystals in a magnesium alloy. Image courtesy Julie Cairney, University of Sydney.

How do I get a good image? - Instrument alignment - Getting started with instrument alignment

Imaging in the TEM requires that the instrument is first aligned so that the electron beam passes down the centre of the column. Just like in other forms of microscopy, the beam also needs to be corrected for any astigmatism, and the focal point set correctly. The following sections explain the basic instrument alignments needed before taking an image.

Condenser lens centre

The first component to consider is apertures. There are a number of movable (adjustable) apertures in the TEM column. The condenser lens aperture, situated below the condenser lens apparatus, can be used to reduce the spot size of the beam and reduce aberration. The condenser lens aperture must be centred along the electron column as shown in the image below.

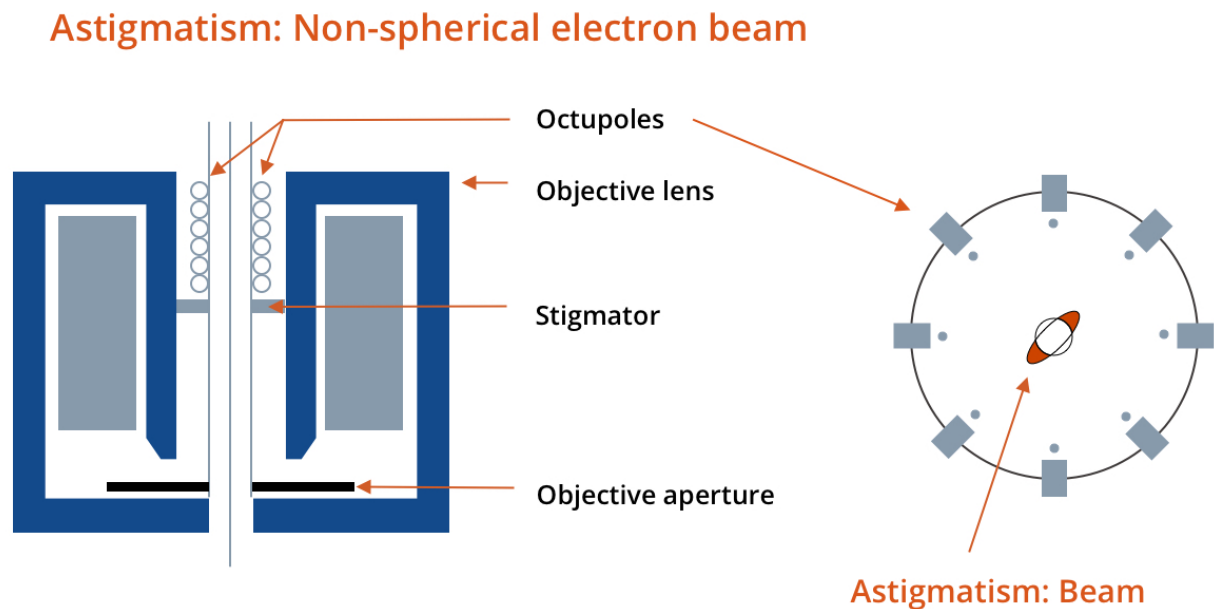


Condenser lens stigmatism

The next alignment to be carried out is to ensure the beam coming through the condenser lens aperture is circular, rather than an oval shape. If the beam is not circular it is said to have an astigmatism. The aberration called astigmatism occurs when the electrons in the primary beam are exposed to a non-uniform magnetic field as they spiral round the optic axis. Astigmatism has several causes. It arises because the soft iron pole pieces that comprise the electromagnetic lens cannot be fabricated with perfect cylindrical symmetry. The soft iron may also have micro-structural inhomogeneities that cause local variations in the magnetic field strength.

The apertures introduced into the lens may disturb the field if they are not precisely centered around the axis. Furthermore, if the apertures are not clean, contamination causes charge accumulation and deflects the beam in unexpected ways.

There are a variety of factors that contribute to forming an astigmatism. These are small octupoles that introduce a compensating field to balance the inhomogeneities causing the astigmatism. Stigmators are present both in the illumination system (condenser lenses) and in the imaging system (objective lens).

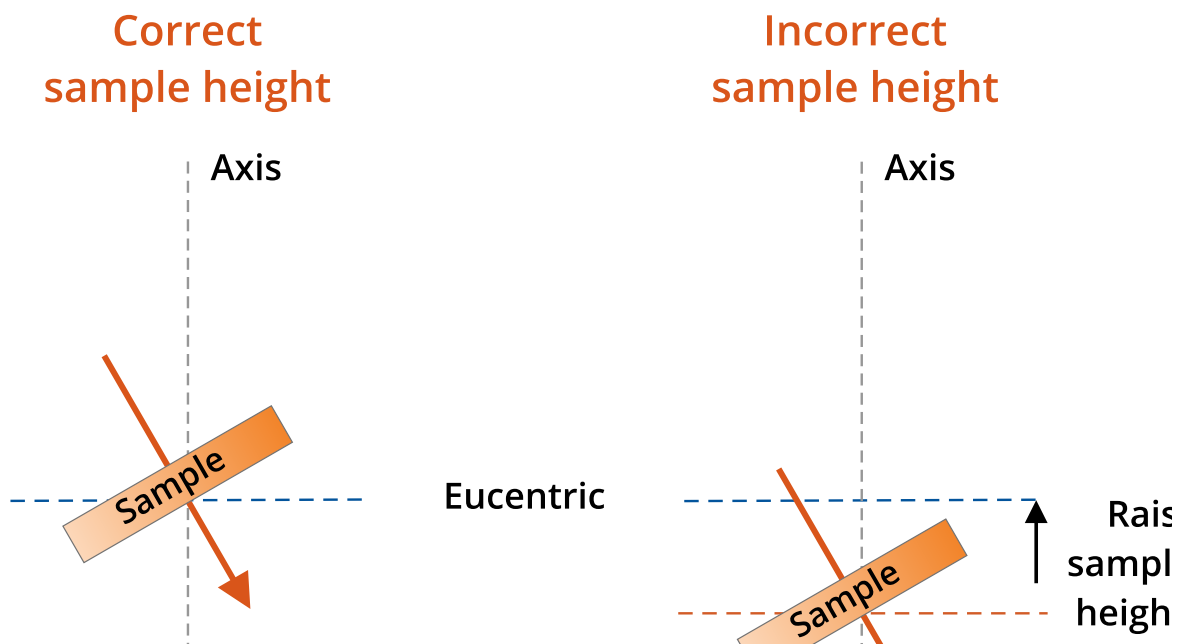


The eucentric position

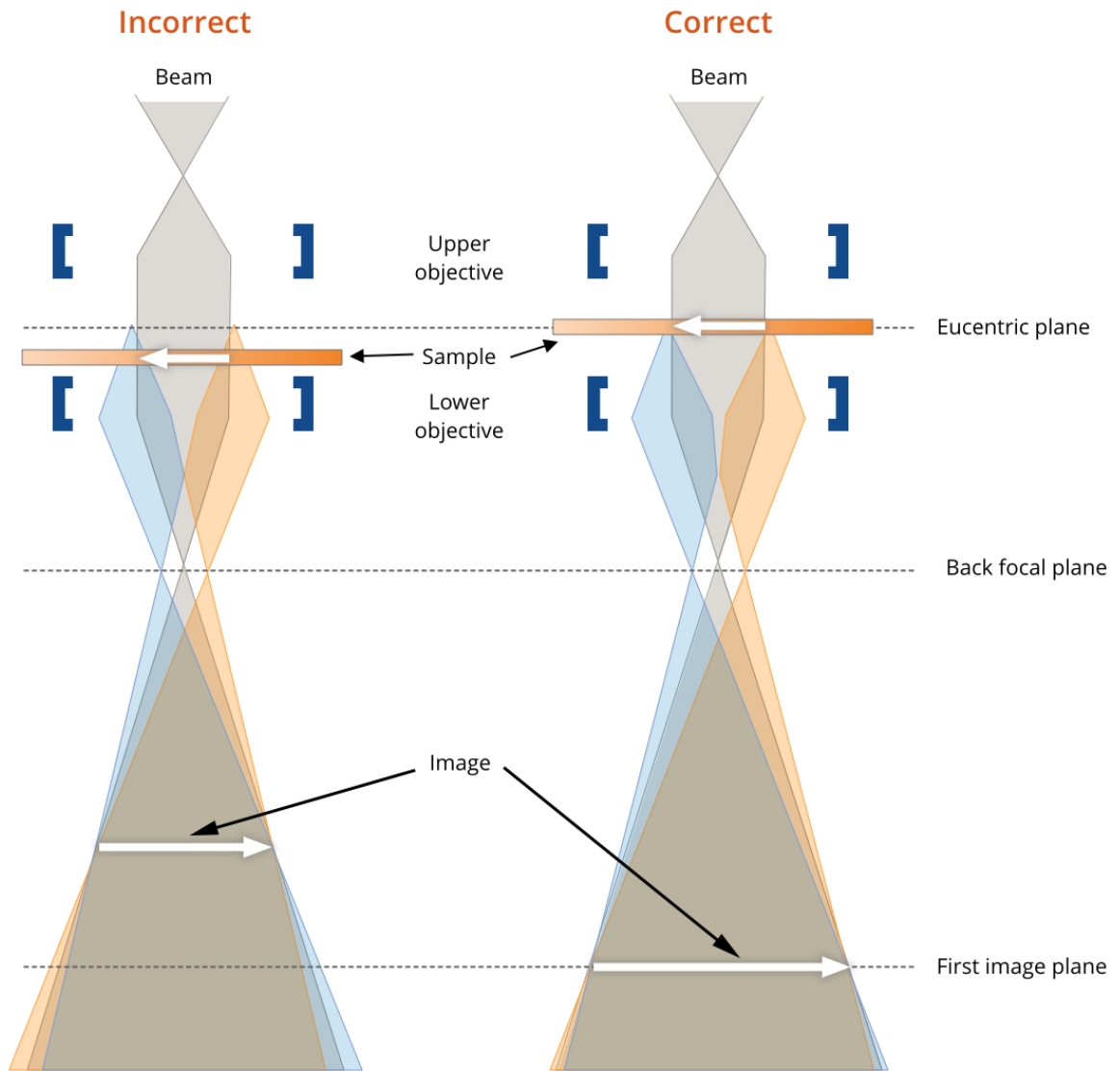
The eucentric position is the horizontal center of the objective lens. The sample must be set to this position. To do this the entire sample holder is raised or lowered.

TEMs are set up so that magnification, camera length, and correct focus are set to this reference position. When the sample height (in the Z-direction) is set at the eucentric position, the sample can be tilted around its axis without the image of the sample moving across the projection screen.

The eucentric height can be set by adjusting the objective lens current to a specific known setting for a specific voltage (e.g. 80kV, 100kV or 200kV), and then raising or lowering the Z-height until the image is in focus. The "wobbler" button can be used as a focus aid where you bring the vibrating split image together into one static image.



A schematic illustration of the correct and incorrect specimen location. Note the focal point of the image is different depending on the specimen location.



Focus

Achieving correct focus of an image is important. When the beam cross-over point is slightly above the sample or slightly below the sample, the beam is not at focus. The presence of an artifact known as a Fresnel fringe can be used as a guide to help focus an image. The image below shows this in practice, where the inner fringe denotes an under focused image, and a thicker outer fringe shows an over focused image.

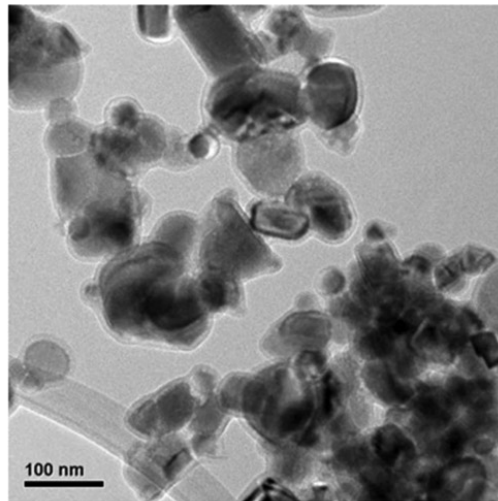
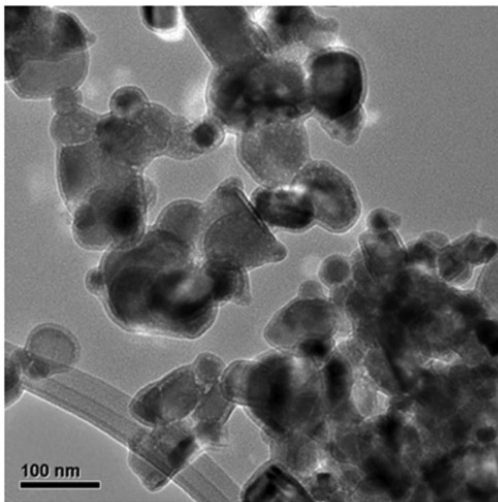
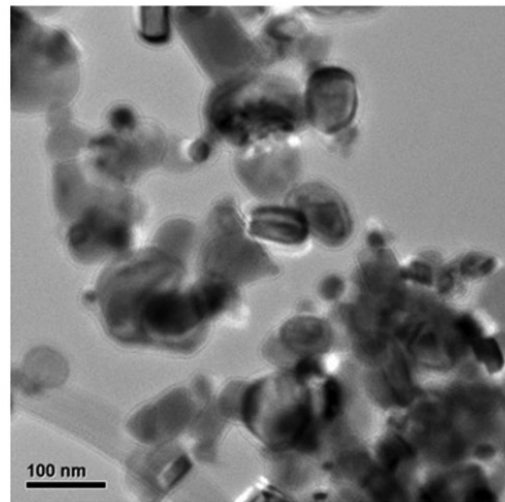


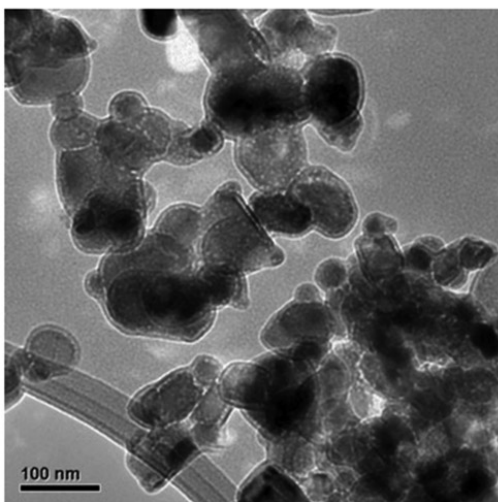
Image in focus



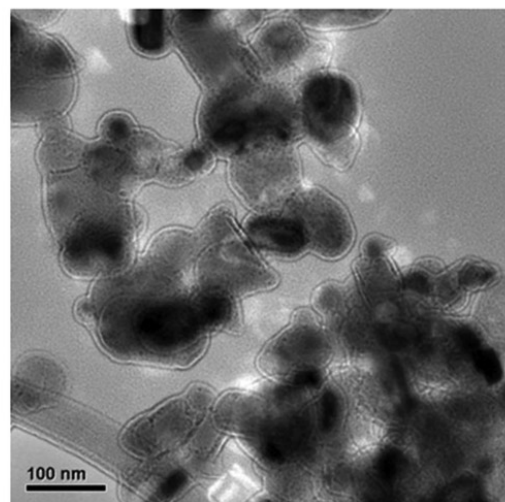
Slightly underfocused



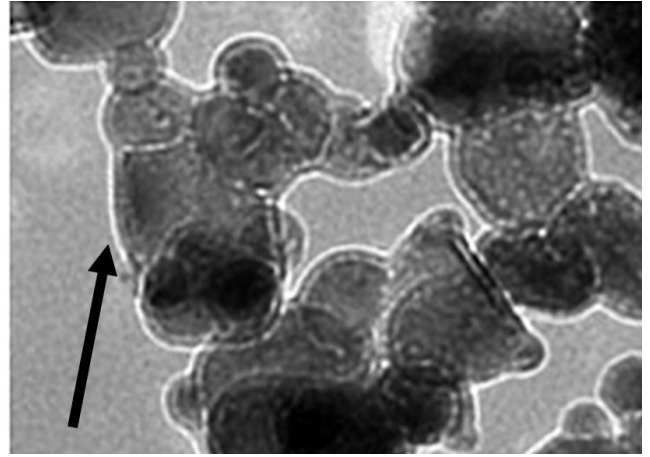
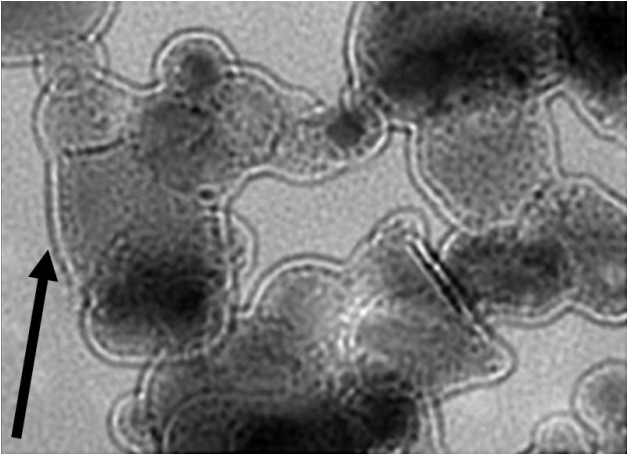
Slightly overfocused



Very underfocused



Very overfocused

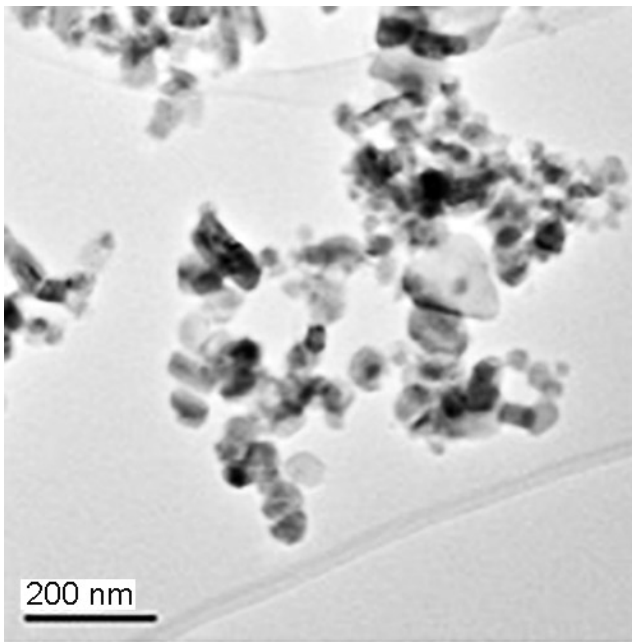


The effect of underfocus and over focus on image quality. Note the bright ring around the particles in underfocus, and dark ring around particles in overfocus. Images courtesy Martin Saunders, University of Western Australia.

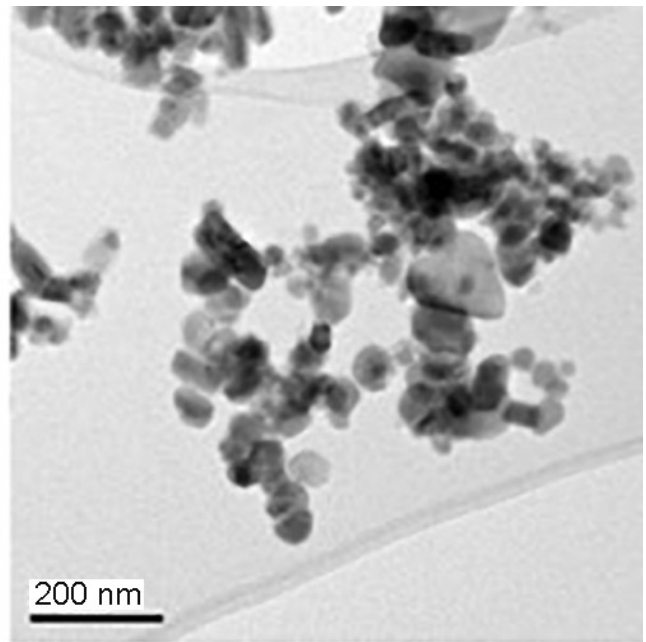
Use of objective apertures

The principle reason for use of the objective aperture is to increase contrast in the specimen. The image on the left was taken without an objective aperture inserted. The image on the right had an objective aperture inserted. Note the increased contrast in the right image. There are a range of objective aperture sizes to choose from: the smaller the aperture, the greater the contrast and darker the image (because more electrons are excluded from the image).

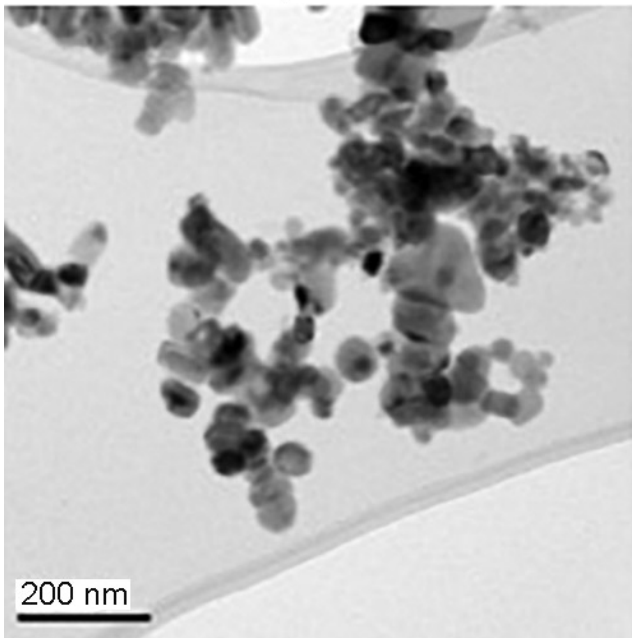
The objective aperture must also be centred along the instrument axis, and astigmatism corrected to ensure the beam is circular.



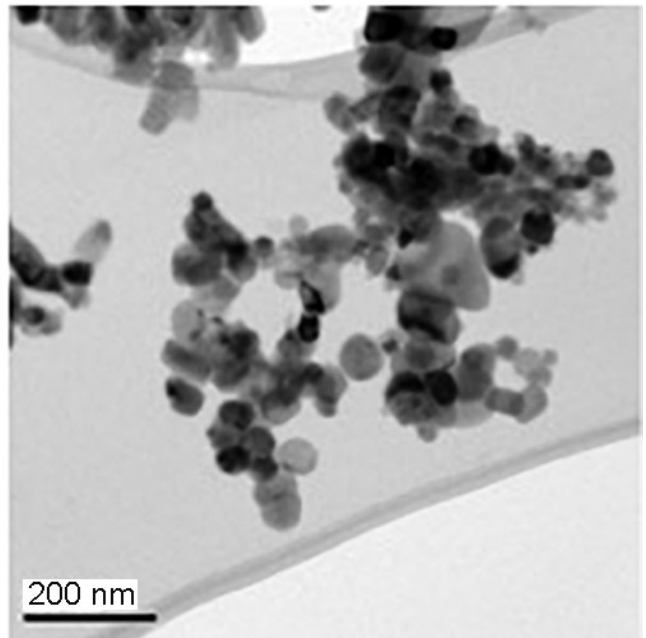
No aperture



Large aperture



Medium size aperture

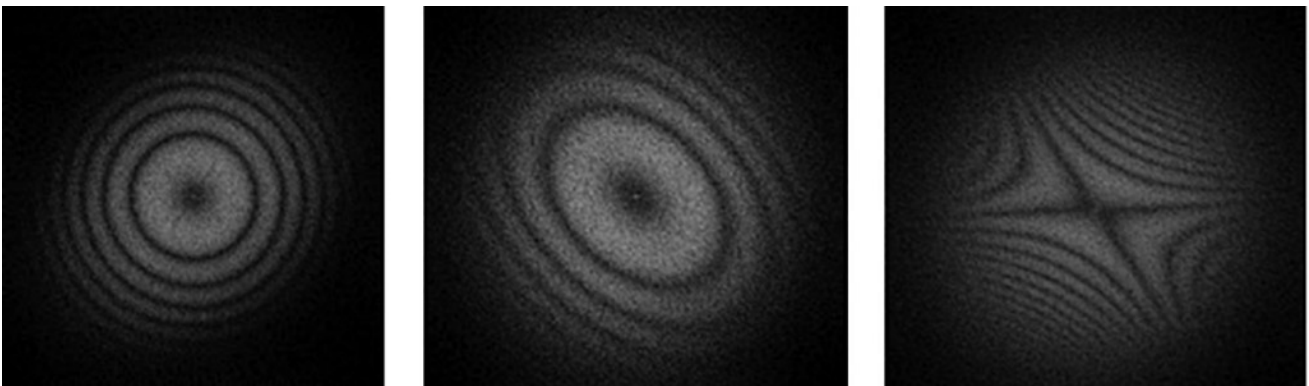


Small aperture

The effect of objective aperture size on image contrast. Note higher contrast for the smallest aperture size. Images courtesy Martin Saunders, University of Western Australia.

Final adjustment

Once the instrument has been aligned with the chosen apertures, a final focus and objective lense astigmatism correction is performed. There are two main ways to do this final adjustment, either optically, as discussed above (by using binoculars attached to the TEM to observe the viewing screen) or by using the Fast Fourier Transform (FFT) application available in imaging software packages for TEMs equipped with digital image collection equipment. The image is a series of examples from a version of software that uses FFT. The left panel shows the signal from amorphous carbon with the image in focus; the other two panels are from the same sample image but it is not in focus.



Fourier transform images of amorphous carbon. Left image shows correct stigmation, centre image shows x-stigmation, right image shows x and y stigmation.

Problems with lenses and alignments - Types of problems

Over the last 300 years, glass lenses have developed to near perfection, while electromagnetic lenses remain quite imperfect. There are at least 10 kinds of defects for electromagnetic lenses but we will emphasise the ones that limit microscope performance in substantial ways.

There are:

- Spherical aberration
- Chromatic aberration
- Astigmatism

Spherical and chromatic aberrations limit the resolution of conventional electron microscopes. Both these defects are unavoidable when using static rotationally symmetric electromagnetic fields. It is necessary to learn how to use the microscope to minimise them.

Spherical aberration is the most significant in defining the performance of the objective lens.

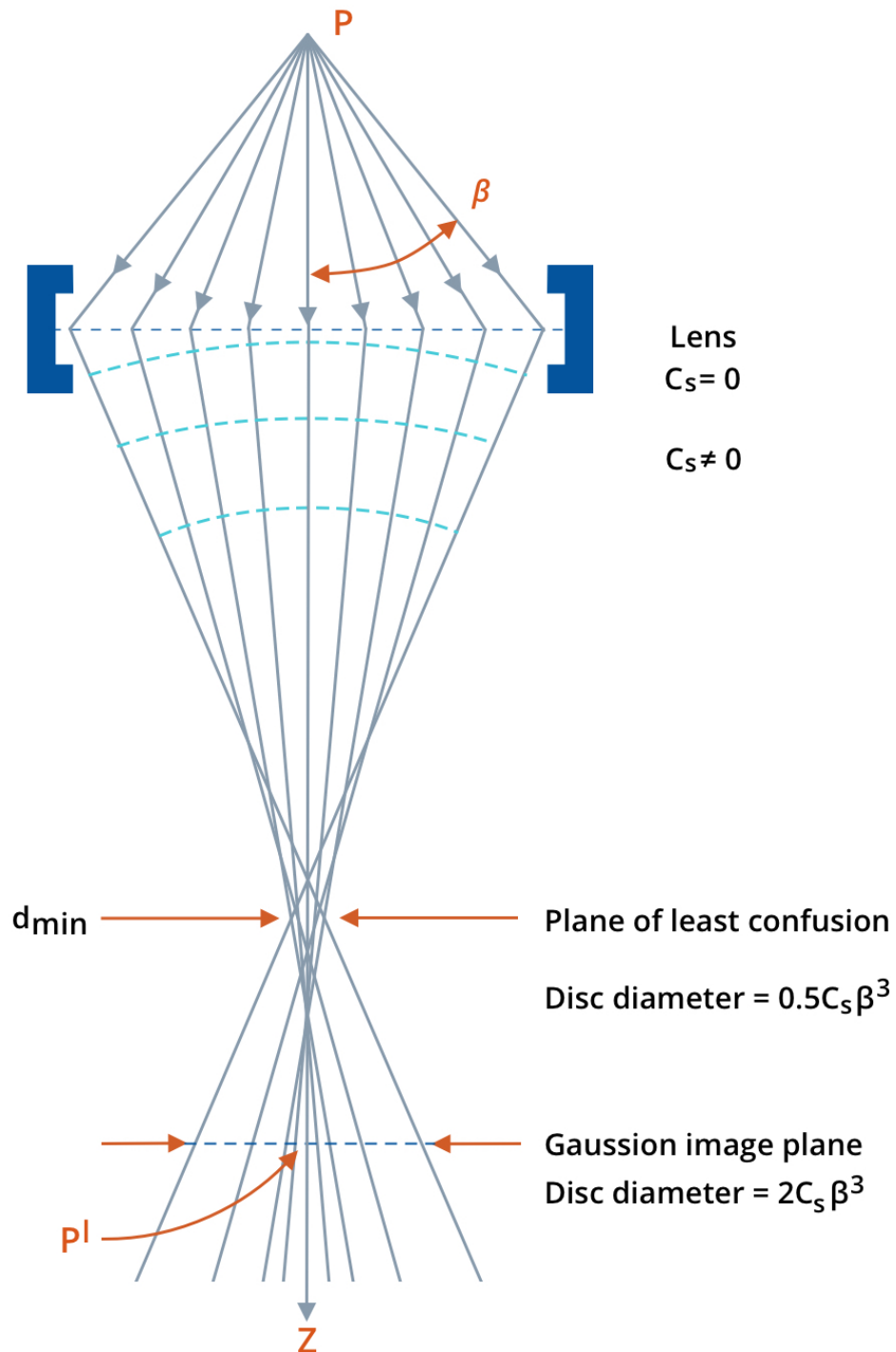
Chromatic aberration worsens for thicker samples. To reduce this problem it is good to make thin samples (e.g. thin foils for physical science preparation).

Astigmatism affects the ability to focus an image but is totally correctable.

Spherical aberration

The spherical aberration is caused by the lens field acting inhomogeneously on the off-axis rays. In other words, the rays that are "parallel" to the optic axis but at different distances from the optic axis fail to converge at the same point. The further off-axis the electron is, the more strongly it is bent back toward the axis. As a result, a point object is imaged as a disk of finite size, which limits the ability to magnify detail, because features are degraded by the imaging process.

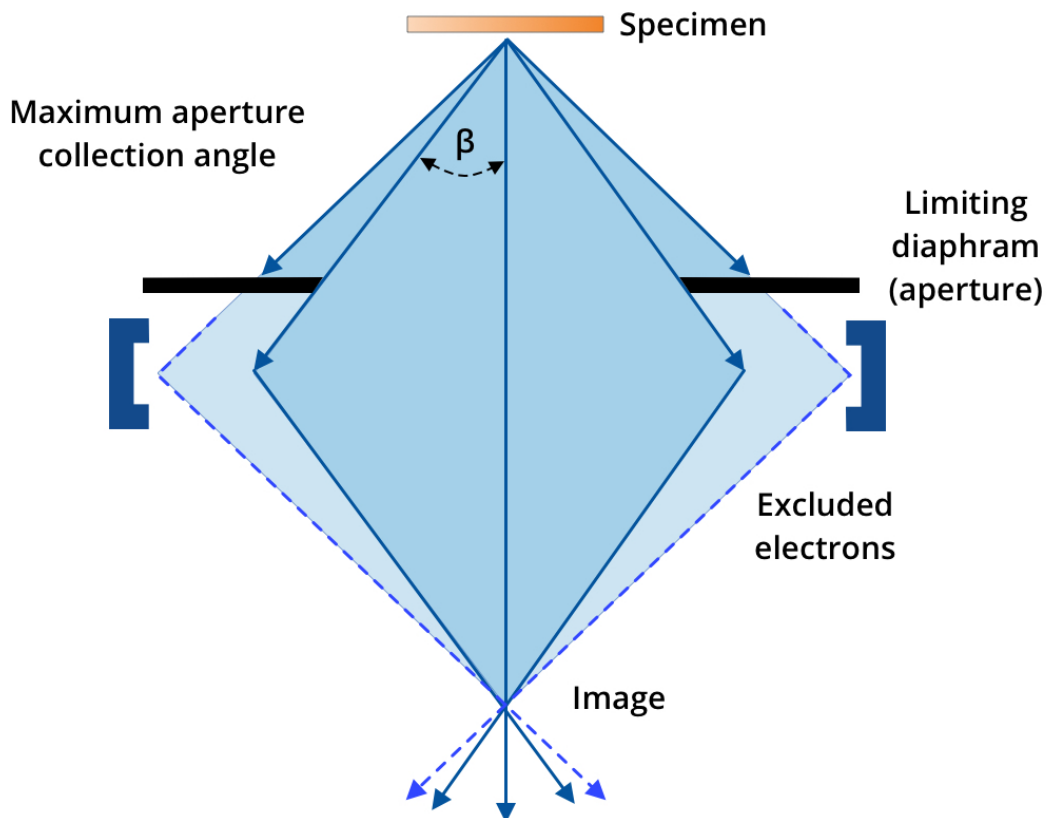
The figure shows the effect of spherical aberration. A point P is imaged as a disk with a minimum radius in the plane of "least confusion" and as PI with an intense central bright region with a surrounding halo in the image plane.



An expression for calculating the radius of the spherical aberration disk (r_{sph}) in the image plane use:

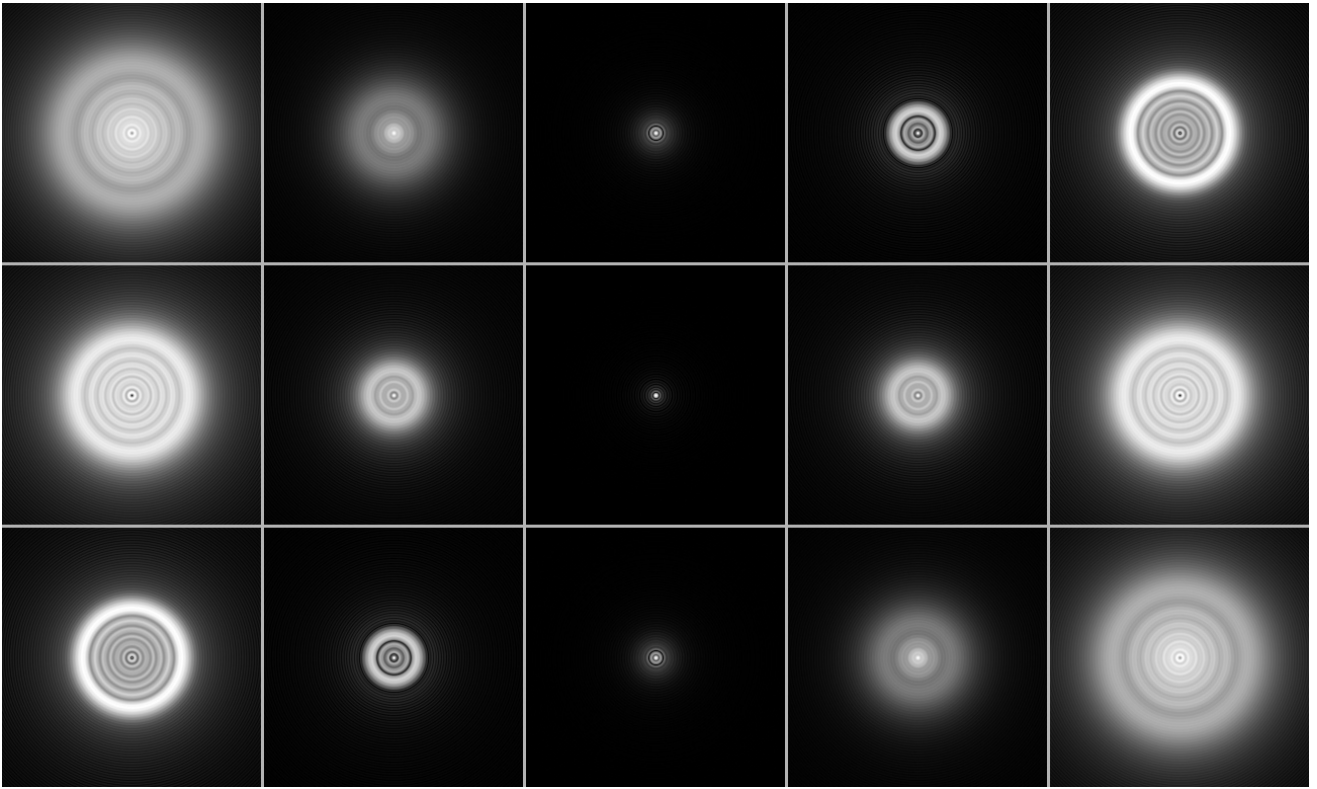
$$r_{sph} = C_s \beta^3$$

Where C_s is a constant for a particular lens called the spherical aberration coefficient and β is the maximum semi-angle of collection of the objective lens aperture. From this derivation, C_s has the dimensions of length; typically, it is approximately equal to the focal length which in TEM is normally about 3 mm but in HRTEM is well below 1 mm. One way to minimise this aberration is to use a short focal length lens (i.e. one with a small spherical aberration coefficient).



This is an example of a point source imaged by a system with negative (top), zero (centre), and positive (bottom) spherical aberration. Notice only the central point is a dot; the images above and below it appear as a disc.

Images left of the centre column are defocused toward the inside; images right of the centre column are defocused toward the outside.



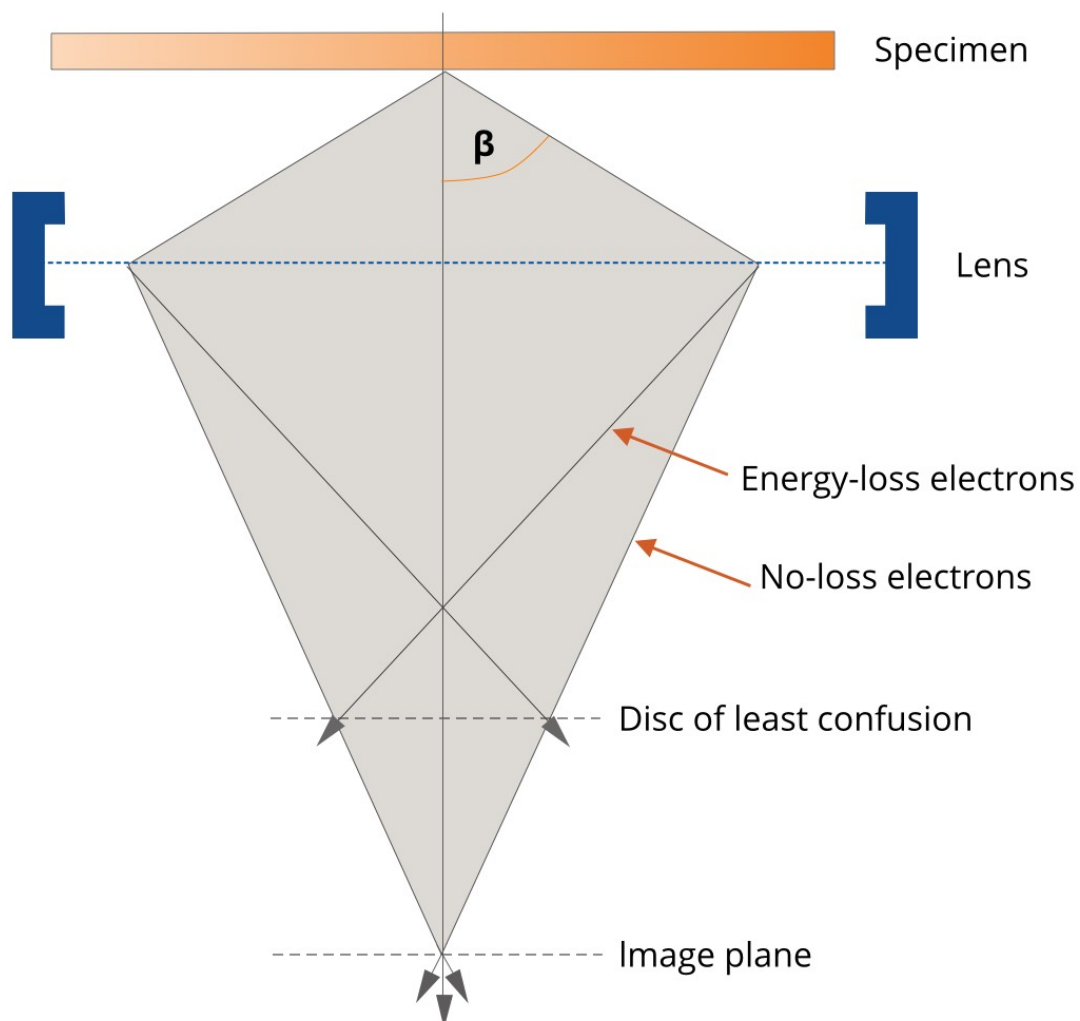
Chromatic aberration

The term chromatic aberration is related to the energy of the electrons.

Electrons are not monochromatic. Electrons emerge from the gun at a whole range of energies and are bent by the objective lens to different degrees; electrons that have lost energy are bent more strongly. Thus, once again, electrons from a point on the specimen form a disk image, as for spherical aberration. The radius (r_{chr}) of the disk is given by:

$$r_{chr} = C_c \frac{\Delta E}{E_0} \beta$$

Where C_c is the chromatic aberration coefficient of the lens (length), ΔE is the energy loss of the e, E_0 is the initial beam energy, and P is the semiangle of collection of the lens. While ΔE in the incident electron beam is < 1 eV. It is typically 15–25 eV for a good fraction of the electrons coming through thin foil 50–100 nm thick. Chromatic aberration gets worse for thicker foils as this leads to a higher fraction of inelastically scattered electrons which may be subject to such effects.



Astigmatism

The concept of stigmatism was introduced earlier, and occurs when the beam is not perfectly circular in cross-section.

Understanding instrument settings

The table below shows the suggested instrument settings for different sample types.

| Thin Sections/ Biological Samples | High Resolution |
|---|------------------------------------|
| 80 - 120kV | 200 - 300 kV |
| Condenser aperture: 1 (biggest) | Condenser aperture: 2 or 3 (small) |
| Spot size: 1 (unless sample is "drifting", 5 or 6) | Spot size: 6 to 8 |
| Objective aperture:3 (smallest) | Objective aperture: 1 (biggest) |

A description of how instrument settings affect the resulting image

An increase in voltage

- = increased brightness
- = increased resolution
- = thicker sections can be used
- = reduced contrast

A smaller condenser aperture

- = increased resolution
- = decreased beam damage to the sample
- = decreased brightness

A smaller objective aperture

- = increased contrast
- = decreased brightness

A smaller spot size

- = decrease in brightness
- = decrease in beam damage to the sample
- = improved sample stability (decrease of sample drift)

Specimen preparation -

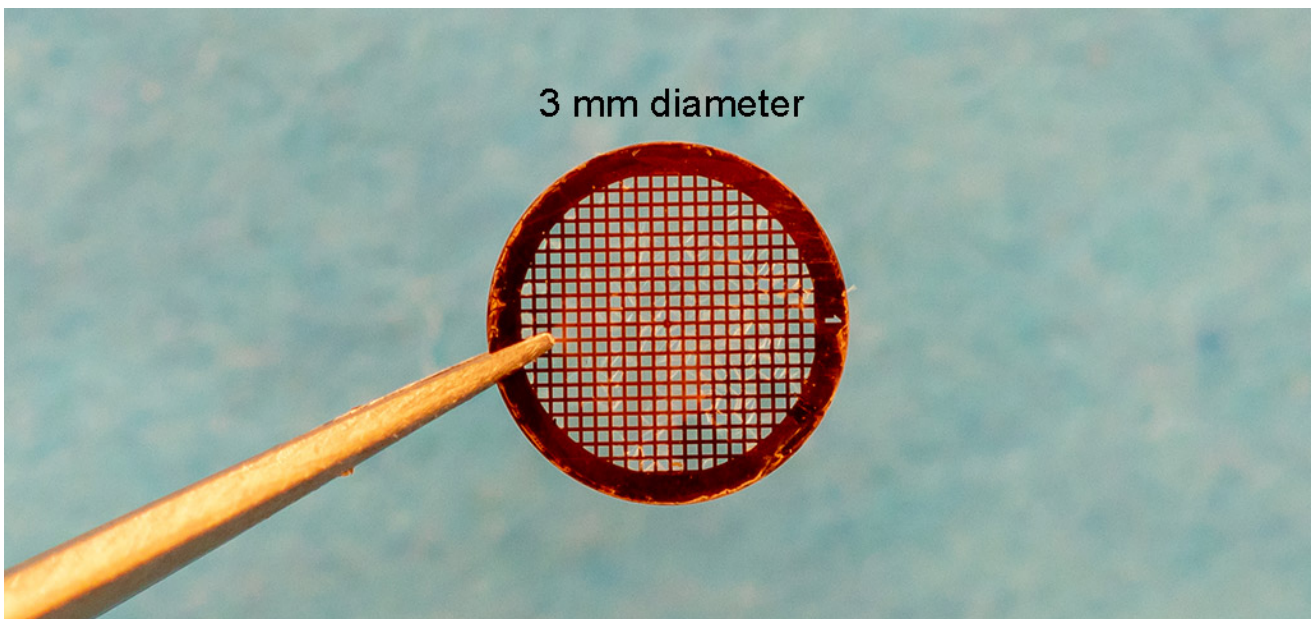
Introduction to specimen preparation

Good sample preparation is vital to getting good quality information from your sample. A poorly processed sample can lead to viewing artefacts. This section is designed to help you choose appropriate preparation processes.

Specimen holders

Sample grids come in different shapes and materials. The most common and cheapest ones used are copper mesh grids. Their outer diameters are always 3mm to fit in the holders and several mesh sizes are available. These can have a square or hexagonal mesh.

Slot grids have a large hole or slot in the middle that needs to be covered with an electron transparent film. The most common film used for mounting physical science powders is a carbon film or a holey carbon film. These films are almost transparent to electrons, so the microscopist can readily view their powder specimens with very little sample preparation required. Although these can be made in the laboratory, most laboratories buy them pre-made to suit their particular requirements. If you are interested in how to make these grids, the following provides a step-by-step procedure for producing holey carbon films, with images: <http://cryoem.ucsd.edu/procedures/H-carbonfilm.shtm>



Copper grid used to hold specimens in the TEM. Image courtesy Dr Nobuyuki Kawashima, University of South Australia

Beyond the goal of retaining the form and composition of the sample in its native state, the main consideration in regard to sample preparation is to obtain a thin enough slice, or preparation, so that the electron beam can travel through the entire sample and out the other side.

Resin polymers are used to support samples: Soft biological or physical science specimens like polymers, clays or particulates are often embedded in a resin polymer before slicing. Particulates can also be handled this way. A hard but somewhat flexible polymer supports the sample and enables thin slices (e.g. 70 nm thick) to be taken from the sample block. If the sample is frozen, there is no need for resin embedding before slicing.

Naturally hard material can be made into thin slices in other ways too [see chart for physical specimen preparation], such as by cutting small blocks then dimpling away the center region, grinding, and polishing, or thinning with an ion beam. This produces a thin disc or foil that does not need further support. But these processes are usually only used for heat-stable or inorganic hard materials such as metals and ceramics.

Particulates can be suspended in a solvent and placed on a support film directly.

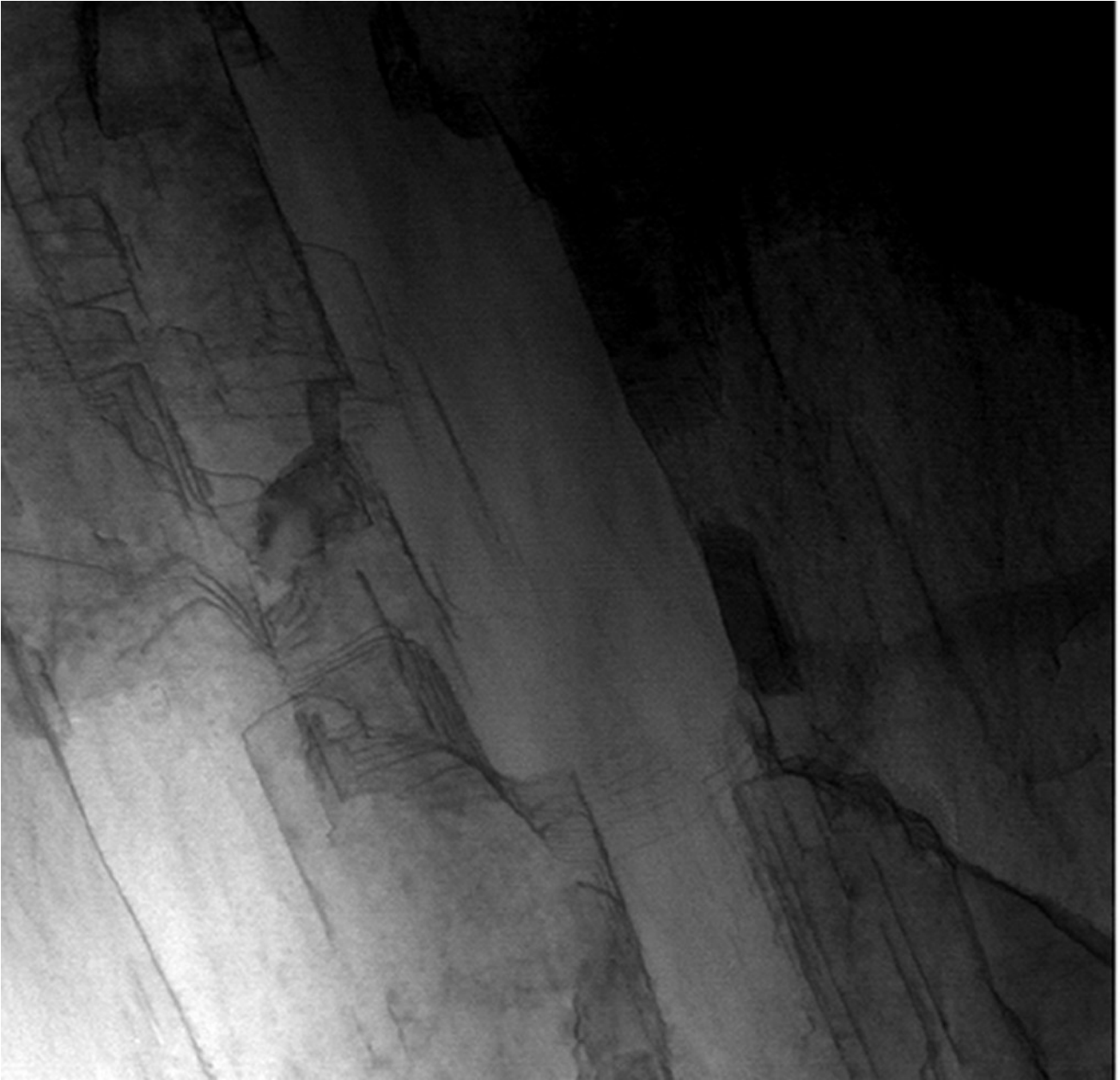


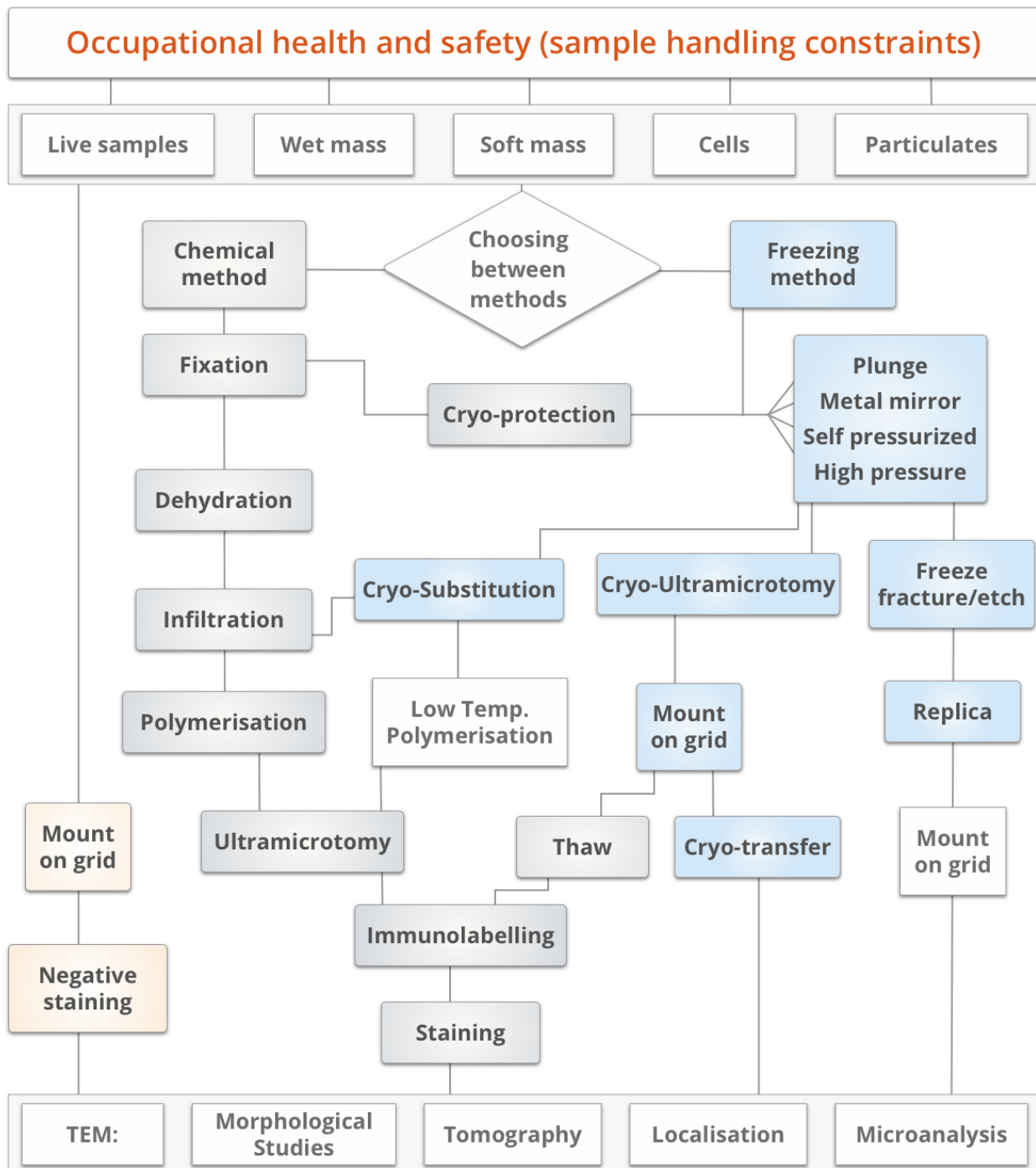
Image shows a poorly prepared specimen with only a small rim of transmission. Image taken from a specimen of hot rolled magnesium, imaged using bright field STEM mode. Image courtesy Nikki Stanford, University of South Australia.

Organic (soft) samples - Getting started with organic samples

Choosing between preparation methods

There are three main types of sample preparation methods that are designed to preserve cellular structures and enhance contrast. These are: cryo fixation, chemical fixation and negative staining.

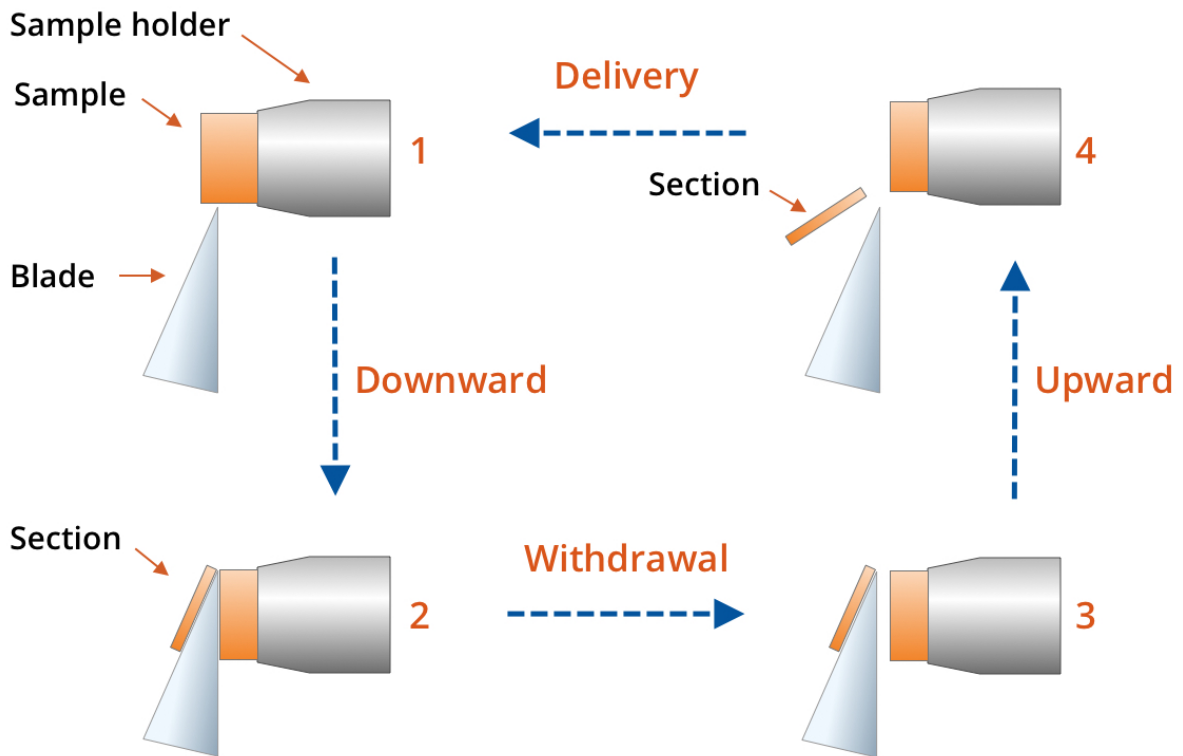
The technique of choice to use on a sample depends on the nature of the sample, the desired end point and the equipment available. Samples that need to be labeled to pin-point the position of proteins (immunolabeling) require a different protocol from samples that are being sliced thinly to look at inherent morphology. When it is desired only to see the outline or surface shape of the sample then a negative staining technique is needed.



Ultramicrotomy

Ultramicrotomy is the name given to taking thin sections off the face of a sample block. The term cryo-ultramicrotomy refers to doing this when the sample is frozen.

A glass or diamond knife is used for the sectioning. At room temperature the sections are usually floated onto a small water bath attached to the front of the knife. If cryo-sectioning is being used then the samples are cut dry and sections are lifted directly from the knife surface as they come off the block.



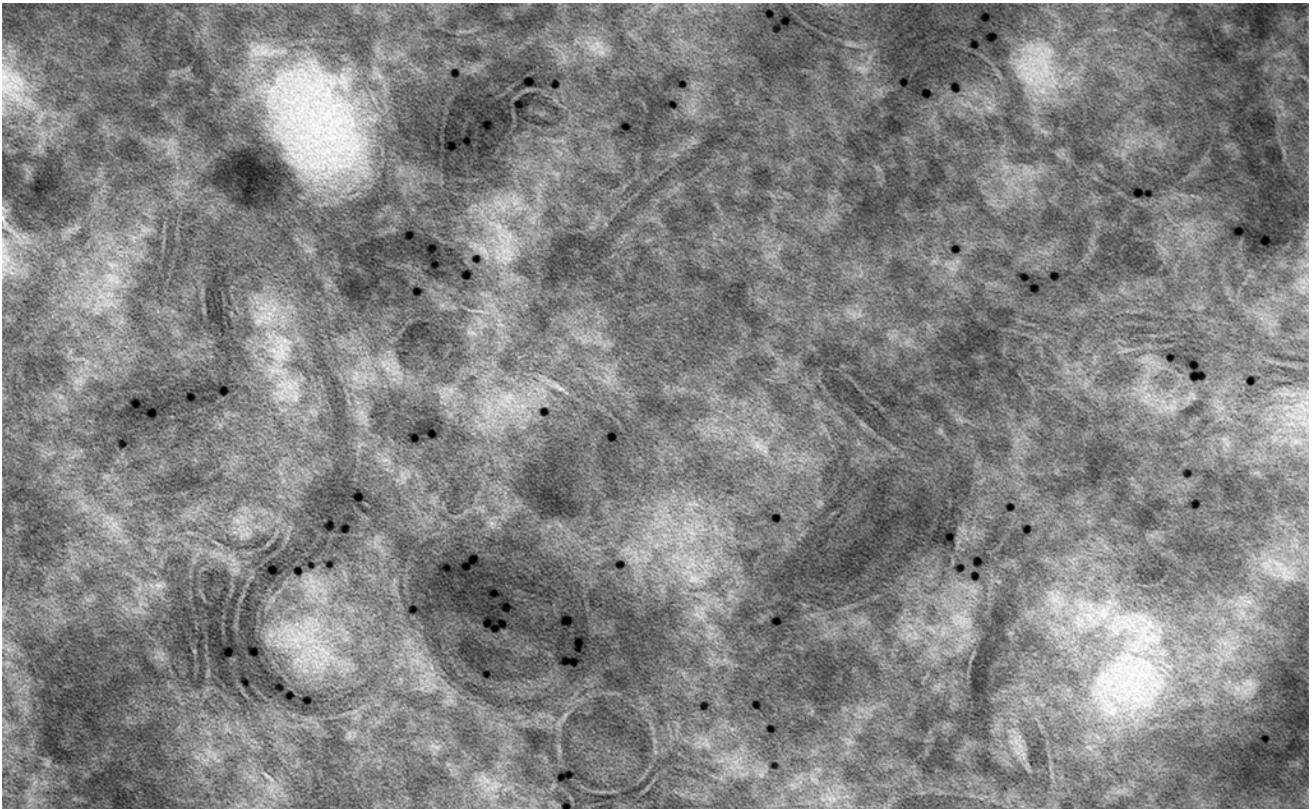
Immunolabelling

Immuno-electron microscopy (immuno-EM; cryo-immunogold technique; Tokuyasu method) is a technique used to detect and label proteins in cells by using electron-opaque beads attached to antibodies (e.g. 10 nm gold beads).

Immunolabeling can be achieved on resin sections and on thawed cryo-sections. The only constraint is that the sample must have retained antigenicity during the processing steps.

The often preferred Tokuyasu method uses cells that are chemically fixed, cryo-protected by immersing in 2.3 M sucrose solution to prevent ice-crystal formation during freezing then frozen and cut to 50-100 nm as cryo-sections. These sections are picked up using a frozen drop of 2.3M sucrose then thawed. The sections on grids are then labeled with a primary antibody that is highly specific to the antigen of interest in the sample. Then a second antibody that will bind with the first is applied. This is the one that is attached to the outside of gold particles (colloidal gold or protein A-gold). Different sized particles are available (e.g. 5 to 30 nm diameter), which allow for double or triple labeling of the one sample with the different sized spheres. The gold can be seen using the TEM.

During the labeling process care must be taken to block non-specific binding with a blocking buffer (e.g. 0.5% bovine serum albumen) and to wash between steps.

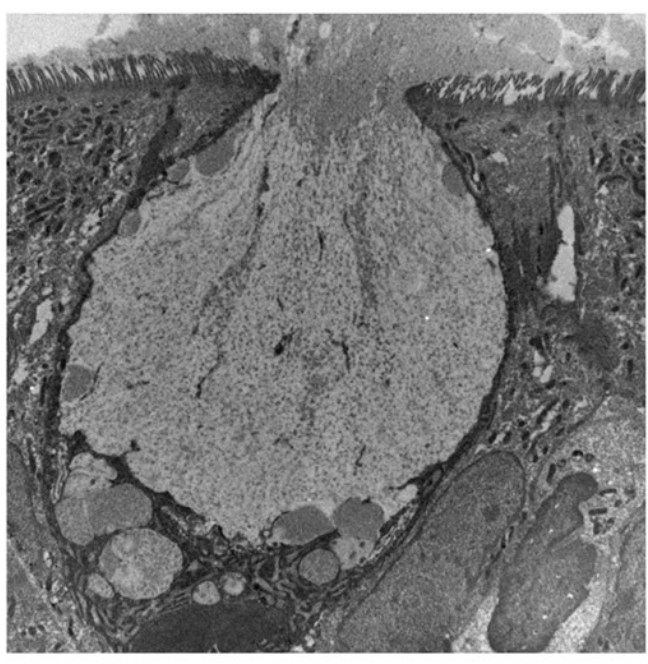
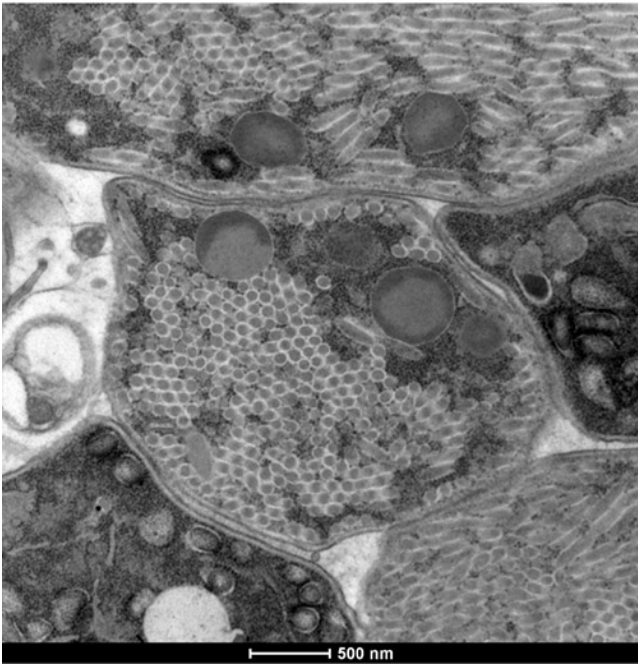
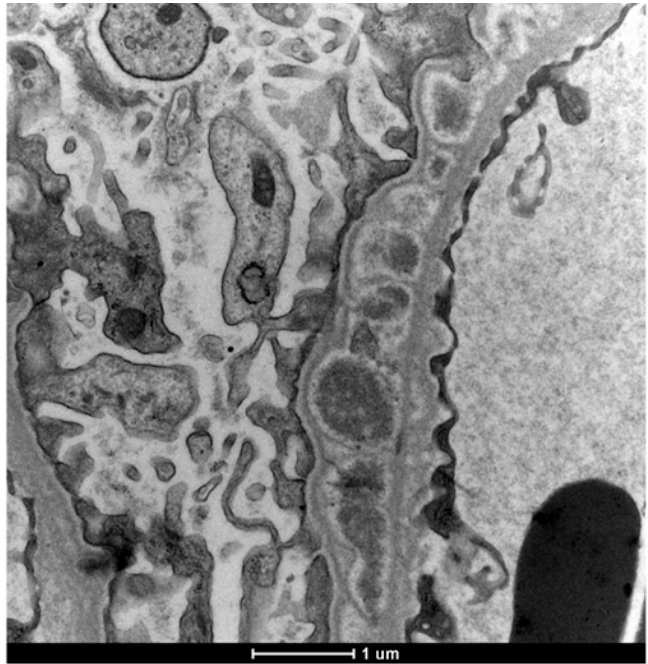
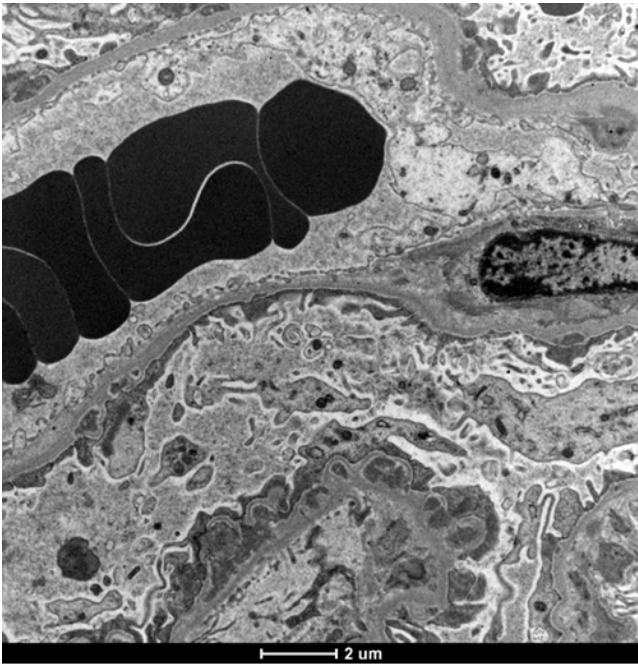


Example of immunolabelled specimen. Labels can be seen as small black spots. Image courtesy Rick Webb and Rob Parton, University of Queensland.

Staining

Image contrast in a TEM comes from the ability of the sample to stop or scatter electrons. It is not from coloured or fluorescent dyes as occurs in much light microscopy. The basic chemistry of cells is made from light elements that do not provide much inherent contrast. Heavy metals that bind to cell structures can provide the necessary additional contrast. Uranyl acetate and Reynolds lead citrate are commonly used stains for this purpose.

Uranyl acetate can be used to stain whole tissue before dehydrating, embedding and polymerisation. However, the more common approach is to stain the surface of the section after ultramicrotomy to achieve some limited penetration of these stains into the sample.



Top two images show immunoglobulin deposit in the basement membrane of a kidney. Bottom left shows a parasitic cyst, and the bottom right shows a gastric antrum in a goblet cell. All specimens underwent chemical fixation in 1.25%v glutaraldehyde, 4% paraformaldehyde in phosphate buffer, to which 4% sucrose was added, followed by secondary fixation in 2% osmium tetroxide. The samples were then passed through a graded series of ethanols, infiltrated with epoxy resin and the blocks were polymerised in an oven overnight at 60 degrees. The sections were cut on an ultramicrotome at 80nm thickness using a diamond knife, and then stained with uranyl acetate and lead citrate. Images courtesy Ruth Williams, Adelaide Microscopy.

Cryo-fixation

Cryo-fixation suits a range of samples. These may be minute suspended particles that can be concentrated by centrifugation; living material that can be transported alive and dissected or sub-sampled in the laboratory; small biological samples (e.g. microbes, or organisms up to 500 micrometres long); and non-living materials that are soft or contain water and can be produced or cut to appropriate size. Dry, soft polymers can be cryo-processed in large pieces since they will not undergo ice crystal formation.

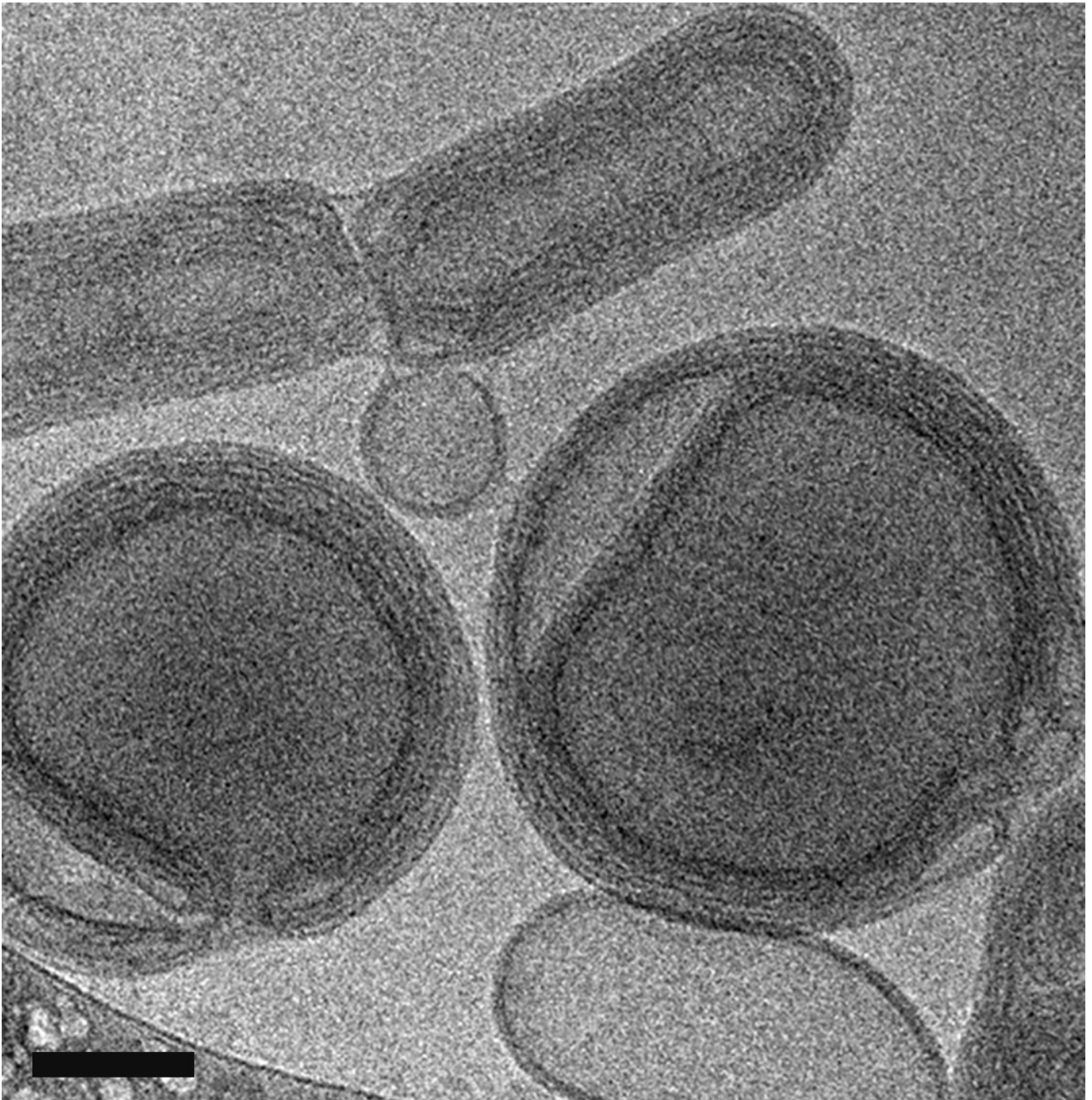
Some samples do not suit cryo-electron microscopy. Samples containing pathogens or blood usually cannot be transported safely from their point of origin unless they are chemically fixed first. Others may be damaged by being cut to a small enough size for cryo-fixation. Postmortem changes may occur if there is a delay between collecting the sample and getting it to the cryo-equipment. This can be an issue with field-caught material or biopsy samples. Some samples are just too big.

While cryo-fixation is generally the best technique, if it is available and the sample is suitable, there is nothing inherently wrong with using chemical fixation and processing. The majority of biological samples are still handled through chemical fixation, room-temperature dehydration, infiltration and polymerisation of surrounding resin, because of the ease of use, the ready availability of chemicals, and the unsuitability of the sample for freezing.

Specialist holders: It is worth noting that there are some specialist sample holders/chambers that can handle wet samples or fluids but these are expensive and their use is rare at present.

Specialist holders:

It is worth noting that there are some specialist sample holders/chambers that can handle wet samples or fluids but these are expensive and their use is rare at present.



Thin-film cryo fixation of multi-layered liposomes. Scale bar, 100 nm. Credit, Delfine Cheng, The University of Sydney

Chemical fixation

The use of chemical rather than physical processes to stabilize the structure of a sample. Why chemically fix samples? Organic and living materials usually need to be preserved before further preparation for viewing. This process is referred to as 'fixation'. Unless the samples are handled in this way artifacts will be seen as a result of degradation or postmortem changes.

Types of chemical fixation:

- Aldehydes are effective primary chemical fixatives for biological tissues since they cross-link proteins.
- Either formaldehyde or glutaraldehyde (or a mixture of both) is the first fixative used on biological tissue. These cross-link proteins and firm up the tissue.
- Osmium tetroxide is used as a secondary fixative and stabilizes lipid components; cell membranes in particular.
- For the Tokuyasu technique of immunolabeling, a very minimal amount of chemical fixation is applied (low amounts of glutaraldehyde, uranyl acetate).

Safety note:

Aldehydes can fix the researcher's tissues as well as the sample so do not expose skin, eyes or lungs to the vapour or fluid. Osmium vapour can fix the eye and produce blindness. Uranyl acetate is radioactive. Use protective clothing, gloves, eye protection and a fumehood.

Dehydration

Dehydration means removing the water and is carried out once tissue has been stabilised through fixation.

The usual alternative is to replace the water in the tissue with a solvent. Acetone is preferred because it is more miscible with the resin that will be used during the infiltration and embedding step.

Note: samples can be dehydrated in ethanol and then switched to acetone in the last 100% solvent step. This is useful to remember if preparing samples for SEM and TEM at the same time, since ethanol is preferred for the SEM processing.

The more rapidly the solutions are exchanged, the more likely it is that tissue damage will occur during dehydration through shrinkage. For this reason a slow exchange is best over a series of increasing solvent concentrations. For example you can start with 30% solvent then move to 50%, up to 60%, 70%, 80%, 90% and finally to 2 changes of 100%.

The time needed for a full exchange and penetration into the tissue depends on the sample: the smaller the tissue block or sample, the better. Some samples such as plants, and particularly seeds, are slower to penetrate because they have a cell wall and therefore these take longer to dehydrate. The usual bench protocol for most samples would be 10 to 15 minutes per exchange. Problem samples may require a few hours to overnight for each exchange.

A laboratory microwave (e.g. the BioWave microwave) can be used to speed up the process. For dehydration you can use 250 watts and 40 seconds per exchange, with no vacuum needed. An example sequence is 50%, 70%, 90 or 95%, 100% and 100%.

Infiltration

The solvent within the sample needs to be replaced with a miscible resin that will polymerise to a hard, but easy to cut, material. This step is called infiltration.

The aim for biological samples is complete and uniform penetration of the specimen. This is achieved by gradually decreasing the concentration of the solvent and proportionally increasing the concentration of the resin in its fluid form.

The process should not be prolonged unnecessarily because extraction of material from the sample can then occur: resin monomers are good organic solvents.

Protocols are dependent on the sample type and size but an example would require something like:

1-2 hours: 50% resin + 50% solvent

2 hours: 70% resin + 30% solvent

2 hours: 95% resin + 5% solvent

2 hours: 100% resin

2 hours: 100 % resin [or overnight in fresh 100% resin]

All infiltration is best done in vials on a rotating disc or with regular agitation or gentle shaking.

Laboratory microwave-assisted infiltration is much faster than infiltration "on the bench", taking only a few minutes per step.

There are a number of different resins to choose between:

- Methacrylate resins are more water tolerant than others but some require oxygen-free atmosphere to harden. The advantage to these resins is that they can be polymerised with low or no heat.
- Epoxy and polyester resins require heat (e.g. 60°C) to polymerise. Epoxy resins are far superior to other embedding media and are usually chosen for regular use. They contain a resin monomer (long aliphatic or aromatic chain), a hardener (setting agent), a plasticiser (for flexibility), and an accelerator (catalyst). These resins are very toxic so gloves, laboratory coat, eye protection and a fumehood should be used.

Resin kits can be purchased rather than obtaining the component ingredients independently.

Polymerisation

Once the sample is infiltrated with fluid resin, the resin needs to be hardened (polymerised). This is achieved by placing the specimen in a capsule (gelatin or polypropylene) or a rubber embedding mould with a batch of fresh liquid resin. The specimen can be orientated to a chosen position during this step. Such planning can save time during the later sectioning step.

A sample label can be placed in the liquid resin adjacent to the sample. Paper is used, with pencil or a printed label. Do not use pen on the label since the ink is soluble in the resin and will dissolve.

The capsules or moulds are then placed in an oven at 60 or 70°C for 1 to 3 days, depending on the resin used. Do not breathe the fumes that come off the hot resin or out of the oven.

It is important to avoid exposing the polymerising resin to water or high humidity. This can be achieved by placing the moulds in closed, heat-resistant containers in which some heat-resistant water-absorbing granules have been placed.

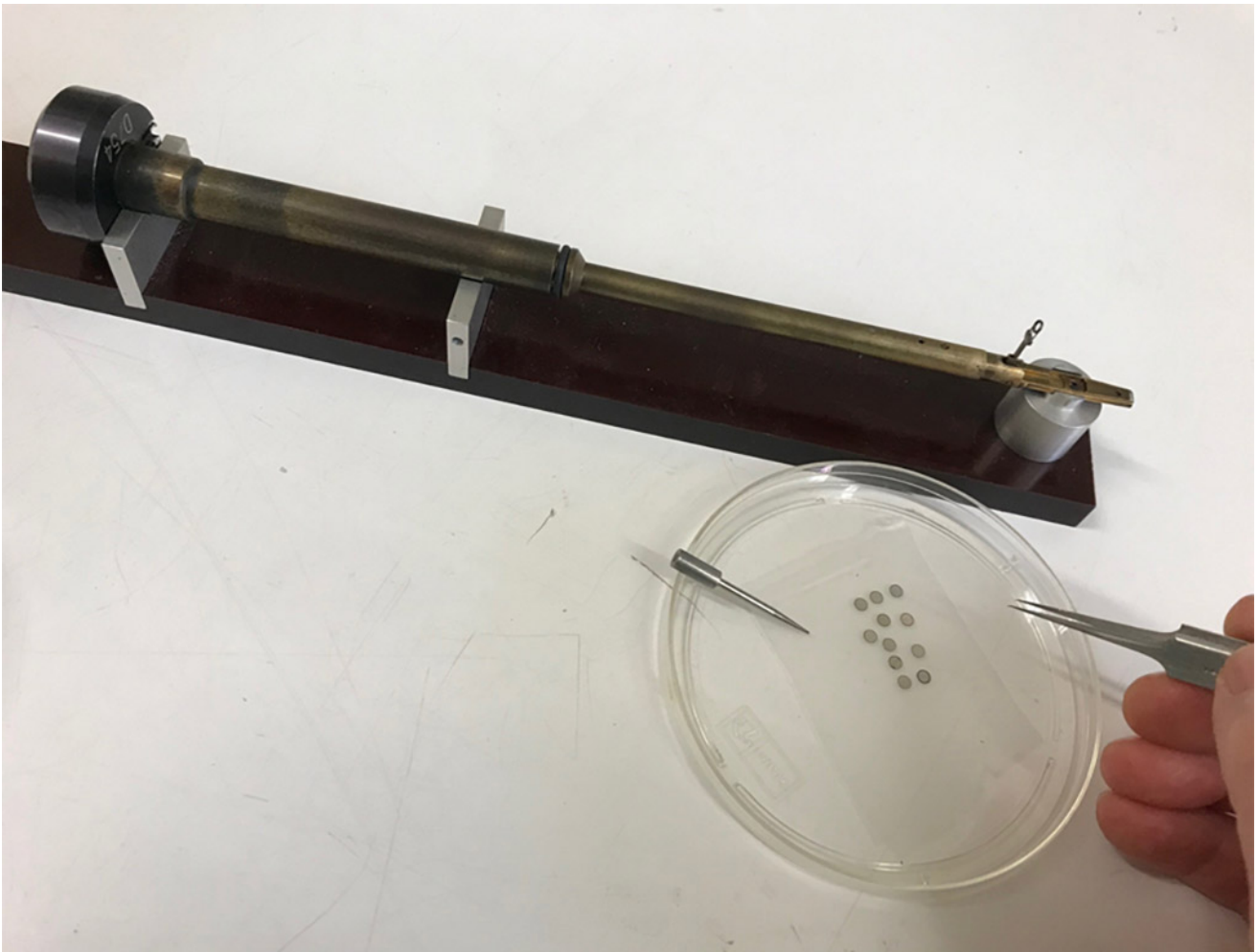
However, some resins can be polymerised under UV light or at lower temperatures. These may require the exclusion of oxygen.

Grid mounting

Samples are usually very delicate and may not be able to hold their shape without the support of a thin film or support grid. Particulate samples also need an electron transparent film to hold them in place. This includes material that is to be negative stained.

Sample grids come in different shapes and materials. The usual ones used are copper mesh grids with a 100 or 200 square or hexagonal mesh. Slot grids have a hole or slot in the middle that needs to be covered with an electron transparent film.

The most common film used is made of formvar (polyvinyl formal) and often stabilised with a fine layer of carbon. Formvar films can be cast on glass and stripped off to float on a water bath, from where they can be applied to the grids. Other materials used for films are plain carbon (usually a holey carbon film) and silicon nitride.



Mounting of a 80 mesh TEM grid in a TEM specimen holder. Credit, Filip Braet, The University of Sydney

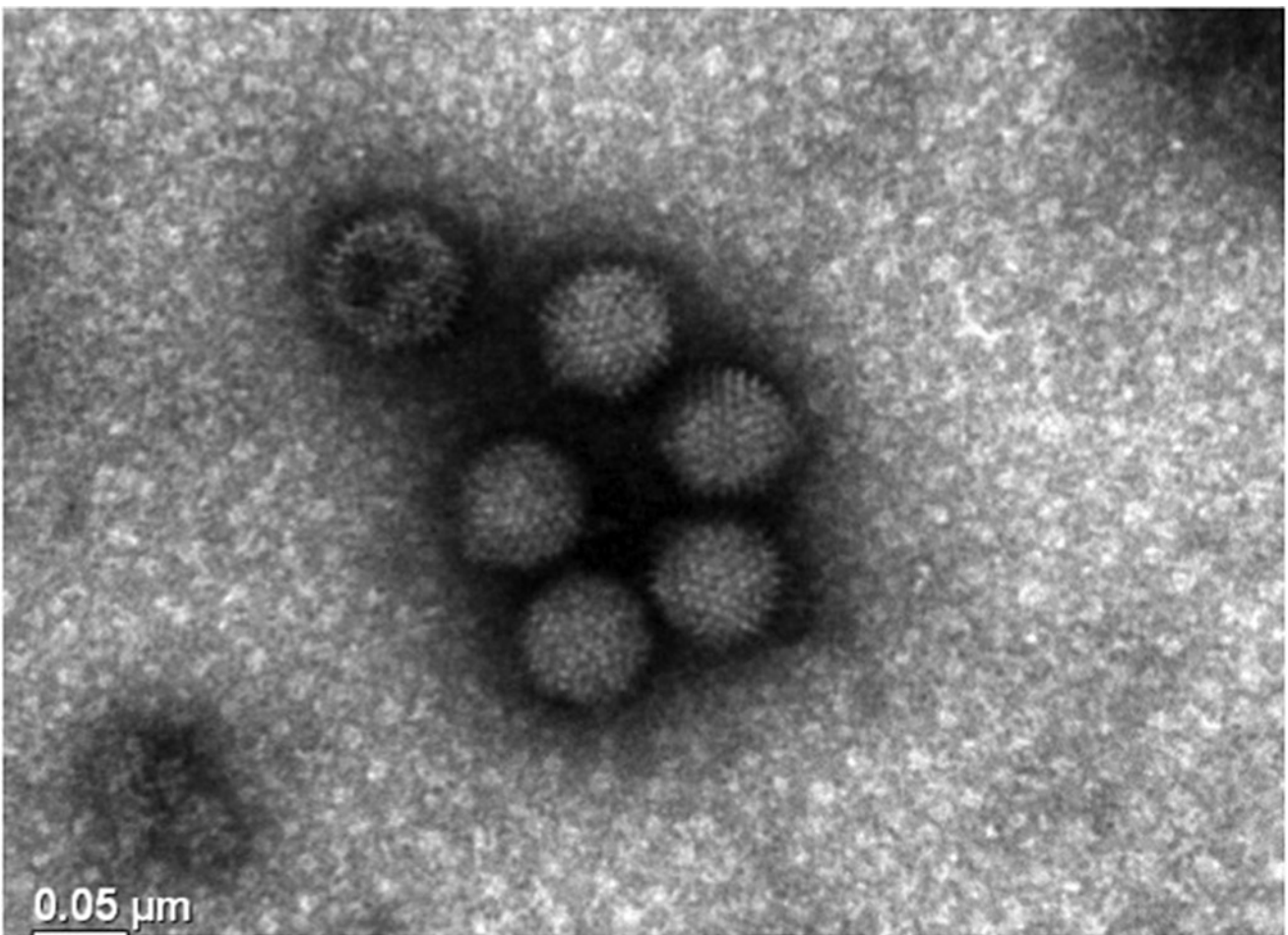
Negative staining

Some minute samples like viruses and bacteria can be viewed by applying an electron-dense stain to the outside. The stain does not penetrate into the sample so no internal detail is seen.

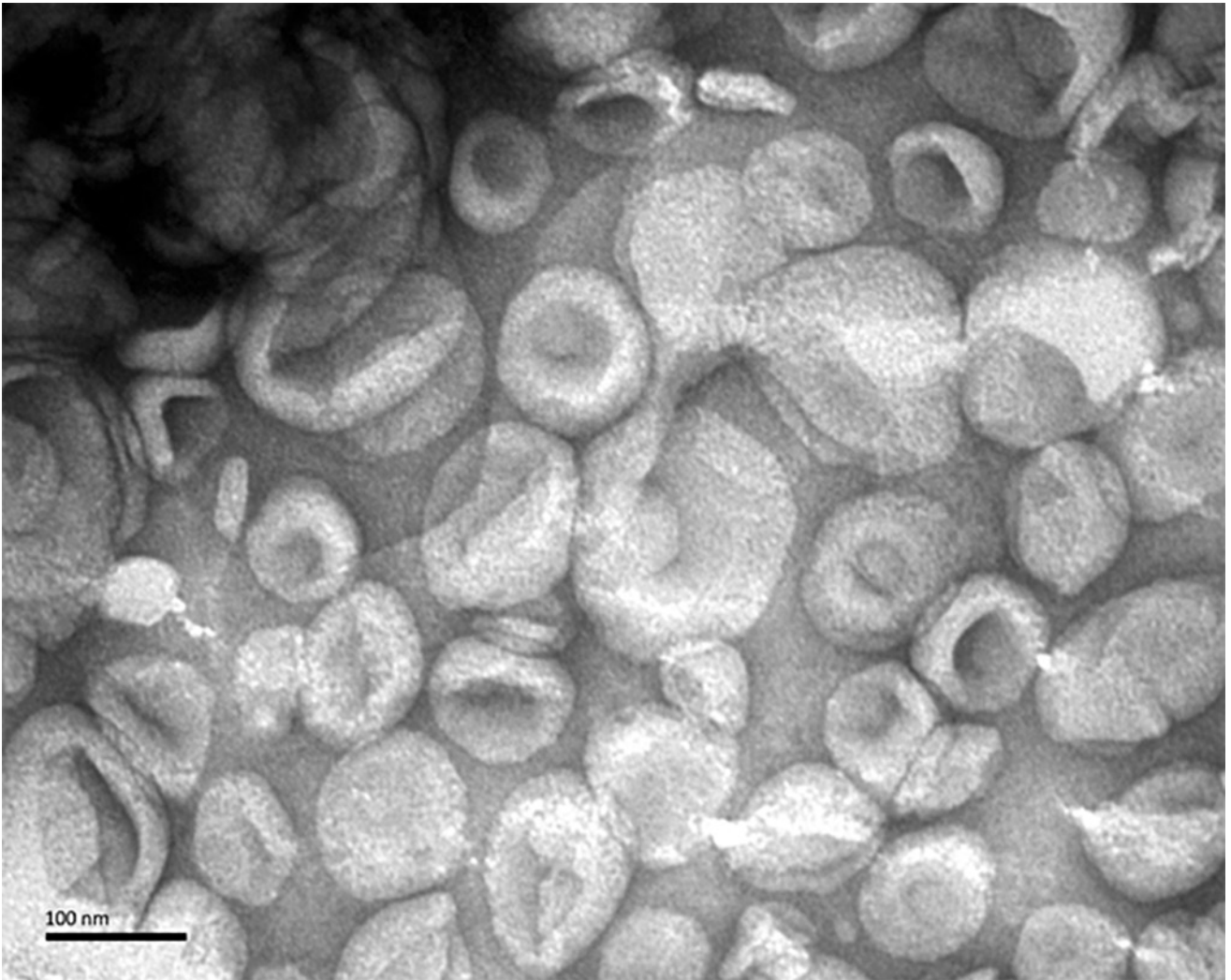
Viewing the external shape can be informative, particularly for macromolecules and viruses and for seeing the flagella of bacteria.

Negative stains used include ammonium molybdate, uranyl acetate, uranyl formate, phosphotungstic acid, osmium tetroxide, osmium ferricyanide and aurogluothionate. These stains adsorb well to biological samples and also scatter electrons well.

Safety: The stains are toxic so it is important to handle them with care, avoiding skin contact, inhalation of particulates and vapours.



TEM micrograph of negatively stained canine adenovirus. Credit: Naveena Gokoolparsadh, The University of Sydney



TEM micrograph of negative stained liposomes. Credit, Filip Braet, The University of Sydney

Cryo-substitution

Freeze substitution, or cryo-substitution, is the process where ice is removed from a sample by an organic solvent at a temperature below the recrystallisation temperature of water. But this is an old definition and there is now some doubt about the second part of it. One must have a frozen sample to commence this process so cryo-fixation is an integral part of freeze substitution.

Once the ice is removed the sample can be warmed, then other procedures like infiltration with a resin can be performed. Cryo-substitution allows the optimal preservation of a cryo-fixed sample to be combined with resin embedding. It gives better preservation of ultrastructure especially avoiding artefacts caused during dehydration at room temperature (such as shrinkage and extraction) plus it gives better retention of antigenicity (for immunolabeling) and retains diffusible substances.

Fixatives can be added to the solvent, starting stabilisation at the lowest temperature at which they are reactive.

Although past processes for cryo-substitution have been time-consuming and involved expensive equipment, there is a simple technique which can be accomplished on a bench with a shaker and a few other materials. The details are published in: *McDonald, K.L. and Webb, R.I. 2011. Journal of Microscopy, 243: 227-233*. The process, as described by one of the authors: Bench cryo-substitution involves the relatively fast and continuous temperature increase from below the freezing point of the acetone to room temperature. In the past the concern that has led to longer protocols has been that of recrystallization during the warming process. It appears that this change from cubic to hexagonal ice may occur without great macromolecular rearrangement, so causing little if any damage to the structure.

The process involves the use of very basic equipment, most of which is easily available:

- A metal heater block with 13mm diameter holes to take cryotubes
- Cryotubes, with external thread but with good sealing o-ring
- An insulated box such as a foam delivery box
- A temperature datalogger, only necessary to initially work out the warming curve for the system [Type EL-USB-TC-LCD]
- An orbital platform shaker, or rocking platform.

It has been shown that a process as short as 80 minutes is enough to get excellent substitution of small samples such as bacteria and tissue culture cells while a 3 hour protocol has given great results with all others samples tested, including difficult samples like plants. The longer protocol has been dubbed "Quick Freeze Substitution" (QFS) and the shorter one "Super Quick Freeze Substitution" (SQFS).

The basic procedure is as follows:

1. Prechill the heater block using a small insulated box filled with liquid nitrogen
2. Add the frozen samples and temperature probe and start the datalogger. Frozen samples are sitting on top of pre-frozen substitution media. Lids must be screwed tightly onto tubes to prevent leakage.
3. Pour out LN2 and place the heater block on its side on top of a shaker platform in a fumehood. Placement of lid can control rate of warm up.
4. Agitate at 100 rpm until the temperature reaches 0°C, about 80 min to 3 hrs.
5. Remove the vials from the block heater, warm to room temperature on a shaker and stop the datalogger.
6. Rinse out fixative with pure acetone and continue on to resin infiltration and embedding.

Addition of water in freeze substitution

Having water in the freeze substitution medium is useful since it enhances contrast. Addition of up to 20% water does not adversely affect the structure. But the common amount added nowadays to the freeze substitution medium is 5% water.

Low temperature polymerisation

Samples can be infiltrated in resin and the resin then polymerised at low temperatures.

Lowicryl embedding resins are acrylate and methacrylate-based and are used for this purpose. They are used at temperatures ranging from -30 to -80 °C.

They have a low viscosity at these temperatures whereas other resins are extremely thick (viscous) and will not infiltrate the sample well. In addition, most other resins need heat to polymerise whereas Lowicryl uses ultraviolet light (360 nm) for polymerisation. The process takes 24 hours.

The reason low temperature polymerisation is used for some samples is to avoid denaturation of proteins and lipoproteins and to retain antigenicity for immunolabeling. [Note: The Tokuyasu method uses cryo-sections that are thawed then labelled directly and does not involve any resin].

It should be noted that a certain level of antigenicity is retained even in samples processed using room-temperature chemical fixation, then dehydration, embedding and resin polymerisation, provided that minimal fixative is used; so low temperature polymerisation is not always essential.

Freezing methods

Cryo-fixation preserves a sample by freezing it at a very low temperature, thus preserving it in as near to natural state as possible. This is a physical process. The aim is to extract the heat from a sample before cell water can rearrange into ice crystals, which destroy the structure of the sample.

The freezing process must be incredibly fast for biological tissues to hold all the dynamic processes going on in any biological system in place and avoid degradation.

Once frozen, a sample can be processed in a large number of different ways, including freeze substitution and freeze drying, it can also be cryo-sectioned or it can be observed directly in a cryo-TEM or SEM.

Plunge/Jet/Spray/High pressure forms of cryofixation

Plunge freezing

Here, the sample is rapidly plunged into a cryogen such as ethane or propane. The depth of good freezing achieved is very poor, though this depends on the sample, but is usually in the range of 10-15 nm.

For cryoTEM work where the thin sample (usually under 300 nm for observation in a 300 kV TEM) is frozen directly on the grid, liquid nitrogen cooled ethane is the cryogen of choice. For samples to be subsequently freeze-substituted, liquid nitrogen cooled propane has been used successfully.

Metal mirror freezing

In this process the sample is slammed against a highly polished copper mirror which is held at liquid nitrogen temperatures. The freezing depths obtained using this are about the same as those for plunge freezing.

In high pressure freezing, samples are subjected to high pressures of 2100 bar milliseconds before they are sprayed with liquid nitrogen. While this gives the greatest depth of good quality freezing (approximately 200 nm) it involves the use of an expensive piece of equipment that is not readily available in most laboratories.

A video is available at the Journal of Visualized Experiments (JoVE) that shows high pressure freezing in action: www.jove.com/video/1943/electron-cryotomography-of-bacterial-cells

Self-pressurised freezing

Here a sample is contained in a sealed copper tube and plunged into a cryogenic liquid. The slow freezing causes formation of large ice crystals in the parts of the sample that initially freeze. This exerts pressure on the remainder of the sample acting in a similar manner to high pressure freezing.

Other techniques such as propane jet freezing and spray freezing are now rarely used.

Cryo-ultramicrotomy

Cryo-ultramicrotomy involves cutting thin sections from the frozen sample block. The sample-holding region of the ultramicrotome is surrounded by a chamber that is chilled using liquid nitrogen to keep the block frozen (e.g. -180°C).

There is a video publication available at the Journal of Visualized Experiments (JoVE) that deals with this topic in detail, showing each step:

www.jove.com/video/1943/electron-cryotomography-of-bacterial-cells

For the Tokuyasu technique of immunolabeling the section is picked up frozen, using a droplet of frozen sucrose (2.3 M), and then thawed at room temperature.

Cryo-transfer

If frozen samples are to be viewed still frozen in a TEM then they cannot simply be carried to the machine and inserted like ordinary samples. This would result in thawing. Even if the sample could be kept cold, ice would condense from the air onto the sample surface. Samples are therefore loaded into a specialist sample holder while frozen and kept chilled during transfer to and insertion into the machine. This involves a contained workstation that can be chilled and tools that can handle the frozen sample.

Freeze-etch

Freeze-fracture or freeze-etch is a preparation technique designed to study the appearance (morphology) of cell contents, in particular lipid membranes and the proteins incorporated in these membranes in a face-on view.

After cryofixation, the tissue is fractured. Such material is easily cracked since it is brittle. Fracture can be achieved by application of force at an edge (via a knife or blade), or by using a cryo-microtome kept at liquid nitrogen temperature.

The frozen fractured surface can be coated in this state or "etched" by increasing the temperature to about -100 °C for several minutes to let some of the ice in the sample sublime off.

Notes are available on the internet at:

<http://www1.udel.edu/biology/Wags/b617/ffe/ffe.htm>

Replica

The frozen fractured or etched surface can have a very thin layer of metal applied from the side to provide a shadowed replica.

Evaporated platinum or gold is applied using a metal evaporator held at high vacuum. The metal source is set at an average angle of 45° to the sample.

A second coat, this time of carbon, is then evaporated perpendicular to the average surface plane. This improves stability of the metal replica coating.

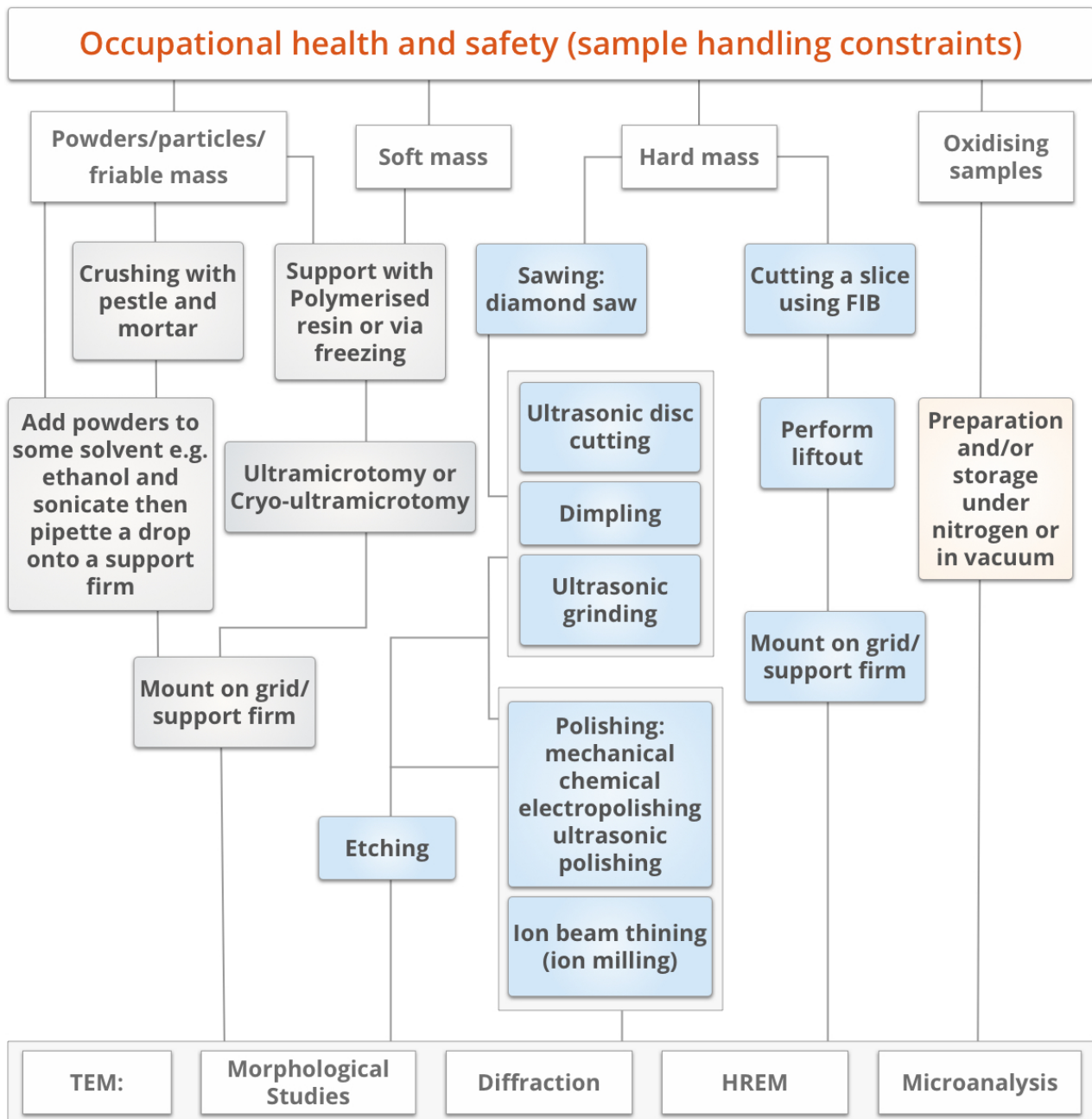
The temperature is brought to room temperature and the pressure returned to atmospheric (from a vacuum status). The very fragile replica is then separated from its underlying tissue by digestion with acids, bleach (hypochlorite) or SDS detergent, and floated off.

After washing, the replica is picked up onto a sample grid and viewed using the TEM.

Notes are available on the internet at:

<http://www1.udel.edu/biology/Wags/b617/ffe/ffe.htm>

Inorganic (hard) samples - Sample prep flow chart

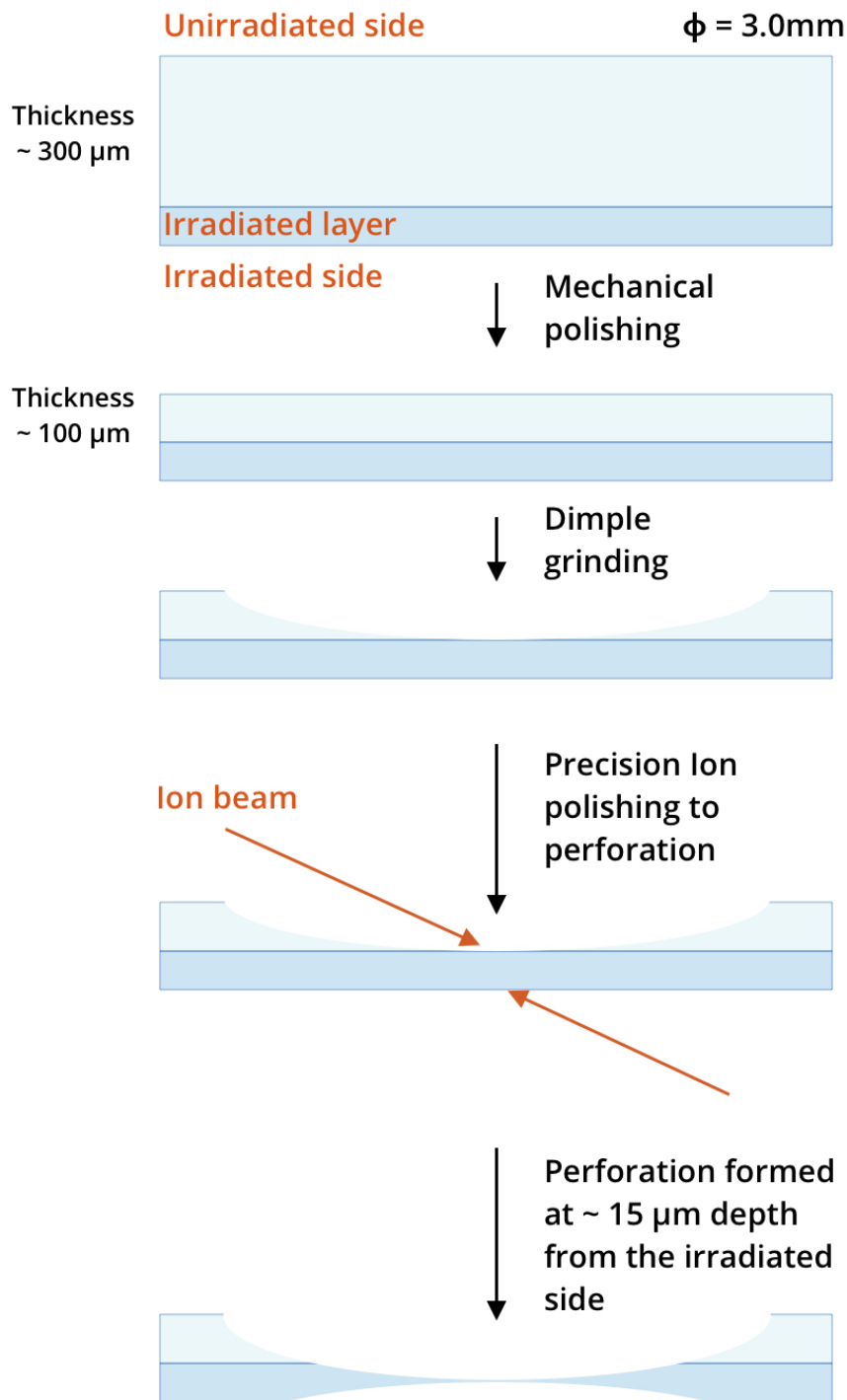


Powders

Bulk friable materials require crushing into small particles or powders before placing onto sample grids. This can be achieved by using a small agate pestle and mortar and adding the material either dry or in a solvent such as ethanol. The pestle is then rocked back and forth on the material to break it up. Once crushed into a fine powder, the powder particles need to be dispersed before applying to a grid or support film. To do this the powder can be added to a solvent such as ethanol and sonicated, or vibrated using a vortex machine, in a closed vial/container for a short time (e.g. 30 seconds to a few minutes). A drop of the suspended material is then pipetted onto the grid/support film. The particles settle to the grid surface and the solvent must be allowed to evaporate fully before viewing in the TEM.

Preparing from the bulk

Hard sample blocks can be cut into slices using a diamond-coated saw blade. As the saw cuts, fluid is sprayed along the cutting edge to act as a lubricant and reduce heat. Use of such a saw will be the start of a process of sample preparation for TEM since the saw cannot produce samples that are thin enough for viewing. They will need further thinning.



Ultrasonic disc cut

A 3 mm disc is cut out from a slice of sample using an ultrasonic disc cutter. The tubular blade cuts down into the sample using vibration and if the disc is not left in place afterwards then it can be popped out from inside the

cutting tube. The disc will then need thinning via mechanical grinding or dimpling.

Mounting the sample in readiness for cutting the disc may require gluing the slice to a metal plate. A low-temperature melting point wax can be used and then later removed with heat. Place the metal mount on a hot plate set to $\sim 70^{\circ}\text{C}$. Apply a small amount of "crystal bond" wax and allow it to melt. Remove the mount from the hot plate and immediately place the sample onto the melted wax on the mount surface.

If the sample is heat sensitive, then glue it to a metal mount with a cyanoacrylate glue (e.g. "Super Glue" or "Crazy Glue."). Later this adhesive can be removed by dissolving in acetone.

The metal mount can be attached to the ultrasonic disc cutter plate magnetically.

Align the area of interest under the cutting tool. Place a small amount of cutting medium onto the sample. Lower the cutting tool to the sample surface. Use a pipette or syringe to wet the powder. Begin the cutting.

Once the disc has completely cut through the sample slice, lift the cutting tool, remove the sample plate and remove the sample by heating the metal plate on the hotplate again or use acetone in a fumehood.

Dimpling

Thinning out the centre of a disc is called dimpling or dimple grinding.

The technique can be used to thin some samples enough for viewing however it is more often used for pre-thinning to near electron transparency and this greatly reduces later ion milling times and prevents uneven thinning.

Bulk samples should be dimpled from both sides. Samples with the region of interest on one surface, (e.g. a thin film on a substrate) should be dimpled only from the "back" side. Measure the initial thickness of the sample before beginning to dimple in order to determine when the dimpling is deep enough.

Grinding paste is used to thin the disc. As thinning progresses use smaller paste. Best results are obtained when a depth equal to three times the size of the diamond grit in the paste is removed by the next smallest grit size. The last step can be used to produce a scratch-free, mirror finish. This is the polishing step shown in a separate box on the sample preparation chart.

The finished disc should ideally be $\sim 300\ \mu\text{m}$ in thickness at the edge, and less than $10\ \mu\text{m}$ thickness in the centre. A single-side dimple should be $\sim 150\ \mu\text{m}$ thick at the edge.

Stop the process often to check the depth of the dimple using the micron scale on the dimpler. Usually if the process breaks through the sample in the centre then the sample will be useless and another will need to be started.

Mechanical polishing

A number of polishing methods exist: mechanical and ultrasonic polishing, chemical, and electropolishing.

Mechanical Polishing

If a sample has been thinned using dimple grinding then the grinding paste can be used to polish the sample, providing a scratch-free, mirror finish.

Mechanical polishing can also be achieved using hand polishing. This is useful when thinning a flat sample or when preparing a sample set at an angle so that the edge becomes thinned to electron transparency. The sample is mounted into a tripod polisher device whose surface can be moved in sweeping and rotational motions across a grit-impregnated disc. The sample position can be adjusted in the holder using micrometer screws.

Cloth discs can be loaded with diamond paste or other grit, or plastic films with different size grit attached, can be purchased. It is standard practice to use smaller grades of grit as the polish is progressively refined. The aim is to achieve a mirror-like surface with no scratches. Developing good polishing skills takes a considerable time investment.

Electro-polishing

This technique is only relevant to metal samples. It uses a temperature controlled bath and a flow of current. The bath acts as an electrolyte. It is usually a concentrated acid or mixture of acids. The anode and cathode are immersed in the electrolyte. The anode (sample to be polished) is connected to a positive terminal of a DC power supply and the cathode is connected to the negative terminal. Metal on the surface of the anode is oxidized and dissolved in the electrolyte. The process of polishing works on the basis that higher topography is removed first.

Ion beam thinning

Ion beam thinning is used to gently remove material from the specimen without inducing damage. This process is often referred to as precision ion polishing, or PIPs. A beam of ions, usually argon, is directed towards at the centre of the specimen disc. The beam is directed at the specimen at a shallow angle, usually $\sim 5^\circ$. If the specimen has been pre-thinned by dimple grinding, ion polishing further thins the specimen until a perforation is made. If the specimen has not been dimple ground, PIPs can still be used to mill the specimen through its entire thickness, but in some cases this takes too long for this to be practicable. For this reason, specimens for ion polishing typically have a thickness of less than 100 μm .

Focussed ion beam milling

Cutting a slice using FIB

A focused ion beam (**FIB**) can be used to prepare thin samples for TEM by a non-mechanical method.

Examples of material that may require FIB are:

- Hard samples that require a thin cross section for observation but are too hard for ultramicrotomy knives.
- Specimens that require preparation of a sample from a precise (to 20 nm) location.
- Material that will be compromised by mechanical cutting, grinding or polishing.

Some samples can be cut relatively quickly with a FIB but preparation of H-bar samples can take 6-8 hours. Mass is eroded out from a trench to leave a thin rectangle and this can then be cut free to become the sample.

Artifacts

Artifacts are often found in TEM specimens. These can be the result of problems with the initial choice of sample or can develop during preparation. Since the aim is to produce a sample for viewing that is artifact free it is important to be able to recognise artifacts and determine what has caused them.

In physical science preparations, artifacts may arise from several sources. For example, during ion milling/thinning, argon ions can be implanted into the sample and this can result in amorphisation and phase transformations. This is caused by using too high an acceleration voltage leading to energetic ions damaging the specimen. Chemical preparation procedures can leave contamination on the sample. Poor mechanical polishing can produce uneven surfaces and scratches, leave residues or introduce dislocations into the sample. Even preparation of particulates on a sample grid can result in artifacts if the samples are sonicated for too long or the wrong solvent chosen. When using cryo-techniques the sample must be able to tolerate the low temperatures involved without compromising the structure.

For biological samples fixation can introduce artifacts. Sample degradation can occur as a result of too much time elapsing between harvesting a sample and fixation. Mechanical damage can be introduced by poor harvesting procedures. Osmotic damage can occur by not matching the fixation solution to the sample conditions. Poor washing between fixation steps can later result in the appearance of “pepper” in the sample from precipitation.

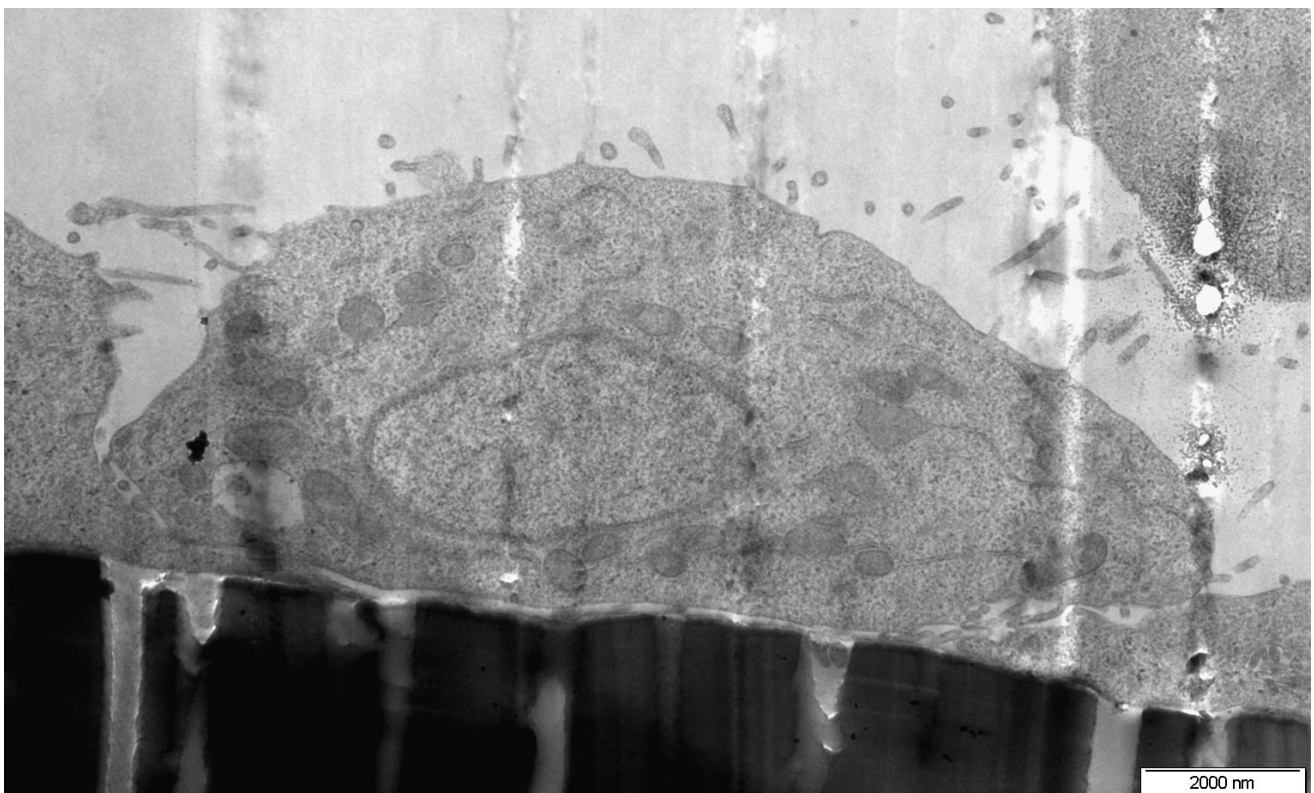
Cryo-fixation has its own range of artifacts related to sample thickness and too slow a cooling rate.

Dehydration and resin infiltration can result in artifacts. Removing water too quickly can result in shrinkage artifacts. Poor resin infiltration and polymerization can result in holes in a sample.

The sectioning of material introduces another set of possible artifacts: tearing, compression, or scratches.

Staining samples can result in precipitation on the surface. Even breathing too heavily on samples during staining with lead citrate will result in lead carbonate precipitation. Poor cleanliness of equipment (forceps) and bench can result in oils and contamination ending up on the sample surface.

Condensing the beam inside the microscope too rapidly onto the sample can also cause damage to the specimen or the film on the grid.



TEM micrograph of colorectal cancer cells with specimen preparation artefacts including tears in the section, staining precipitates and knife marks from sectioning; Scale bar: 2000nm; Credit: Naveena Gokoolparsadh, The University of Sydney

Specialised TEM techniques - Cryo - TEM -

Introduction to Cryo

When we observe samples in the electron microscope, we always have to keep in mind that we bring our samples into the vacuum of the electron microscope column and bombard them with high energy negative charged particles, the electrons.

For most biological samples this is a very hostile environment and biologists go to great lengths to prepare their samples for this environment so that they can be confident that the structures they observe are close to their native state and that a cell is still recognisable as a cell and doesn't look like a grape, that after drying, is transformed into a raisin.

One method to archive this is to snap freeze living cells or cell extracts while they are still in their native environment and observe them in a frozen, but otherwise native state in the electron microscope. While this sounds easy to do, in reality there are quite a few obstacles to overcome in order to get the desired outcome. Not without reason have the pioneers of this field been recently awarded the Nobel Prize.

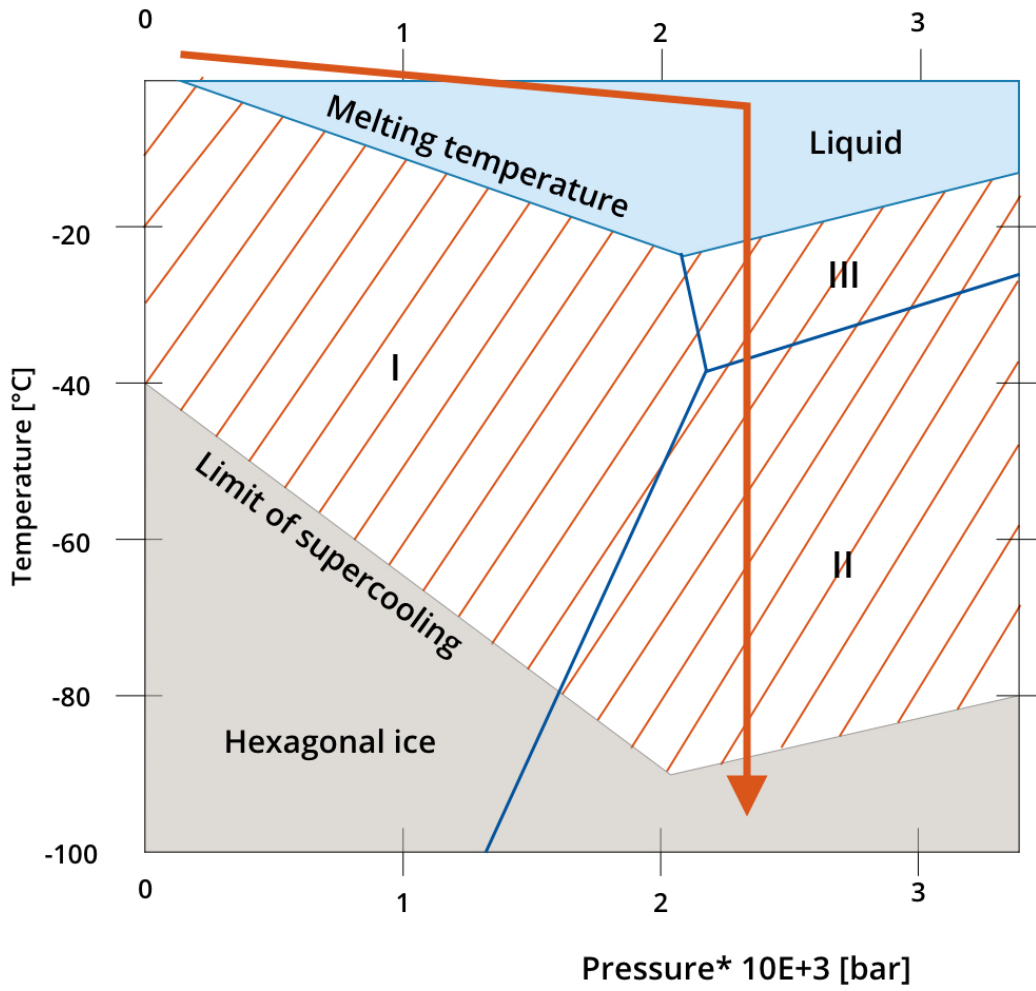
The first obstacle is to freeze the water properly. The conventional way of freezing water is to just slowly lower the temperature until eventually the water solidifies, but what happens during this process on a molecular level is absolutely destructive for cells but also potentially destructive for isolated biological molecules.

Besides local concentration changes of the solutes in the medium in and around the cells during the cooling process, the water eventually forms hexagonal ice crystals and the volume of the water increases by about 9%. The growing ice crystals pierce the cell membrane and rupture cell walls and the local concentrations changes of salts and buffer substances can cause havoc for the finely tuned buffer conditions for isolated macromolecules leading to precipitation and denaturation.

Although not all macromolecules are that sensitive to freezing the presence of ice crystals alone will interfere with the image formation by the electron beam. Electrons can end up diffracting along the crystal planes of the ice rather than allowing the conventional imaging of the samples. The way around this is to avoid formation of hexagonal ice crystals during the freezing.

Water needs to be frozen in a glass like amorphous state, or at least with only very small ice crystals that don't change the density of water and don't interfere with the electron beam in the EM. For biological samples there are two different methods to achieve this.

1. For samples thinner than 0.2 mm, water can be frozen by pressurising it to 2100bar just before freezing it with liquid nitrogen down to a temperature of -196°C. This circumvents the hexagonal ice phase and can be used for cell suspensions or small pieces of tissue, but often needs further processing i.e. by thinning of the sample in a cryo-ultramicrotome. (see also simplified phase diagram of water in slide 2)
2. By cooling water down to -196°C at ambient pressure in a thin layer, but at a speed faster than 10,000°C/s. The last method forms truly amorphous, vitrified ice with no ice crystals.



Modified from Kanno H, Science, 1975

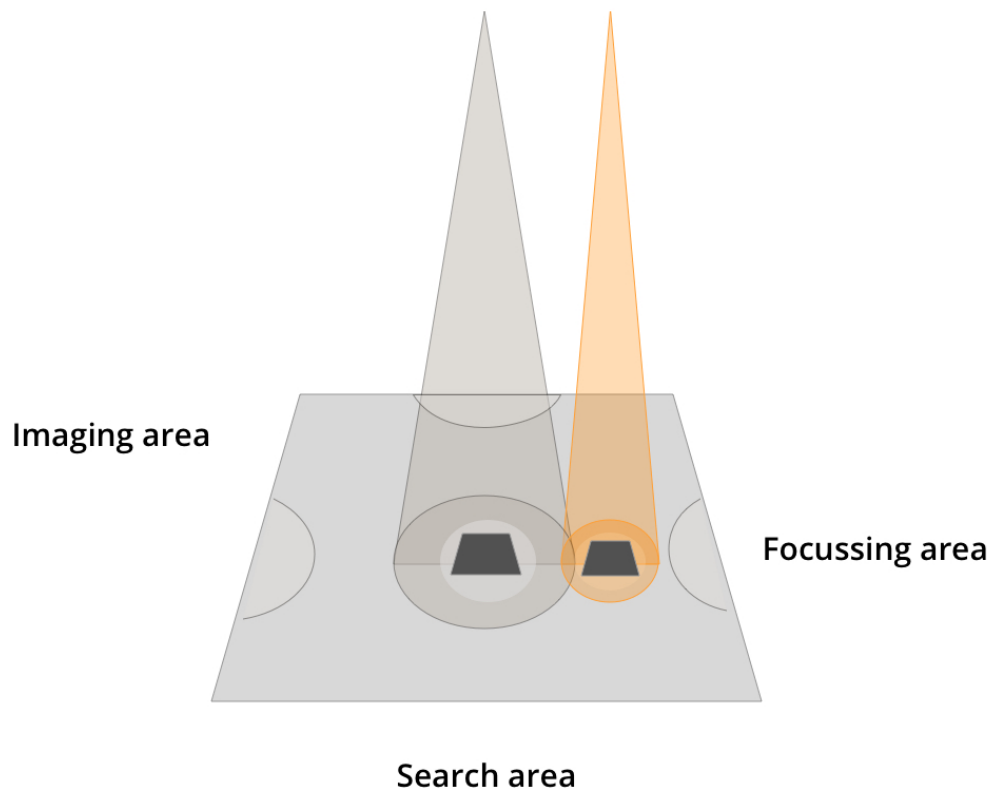
Why Cryo TEM?

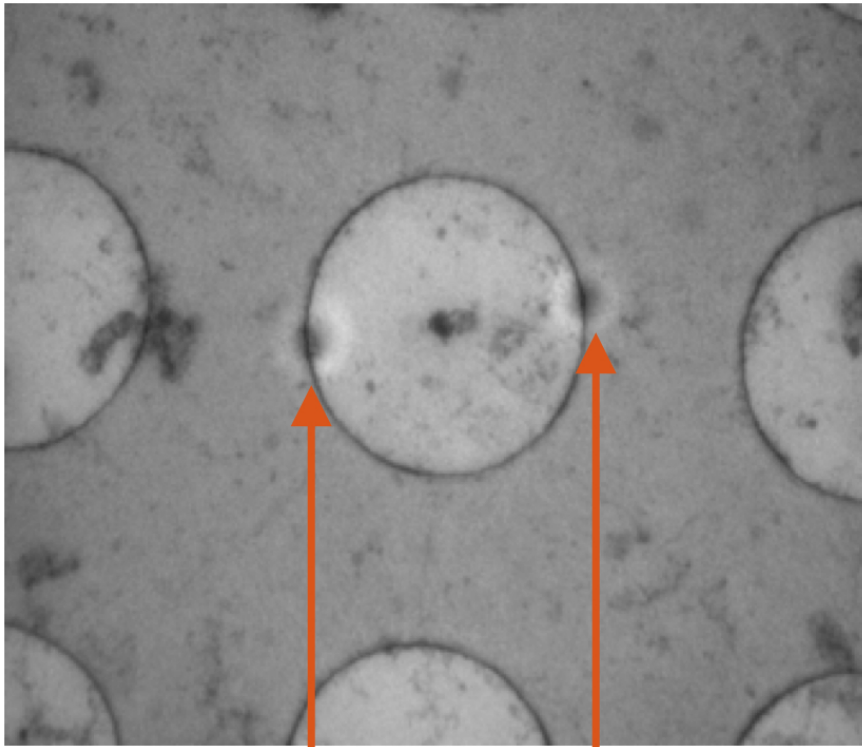
Most biological specimens can't withstand the hostile environment inside the TEM without special preparation. For isolated protein macromolecules the simplest way to do this is to dry the samples on a thin carbon film and contrast them with heavy metals salts like uranyl acetate (negative staining). The disadvantage is that drying and contrasting changes the sample structure.

The solution is to freeze the macromolecules in their native solution in a thin water film over holes in a carbon film. This allows biological material to be safely introduced into the vacuum of the TEM column. They are however, still very beam sensitive. At temperatures of $<180^{\circ}\text{C}$ and at 300kV acceleration voltage beam-induced damage begins at an electron dose of less than $30\text{e}/\text{\AA}^2$. This is much less than dried and heavy-metal-contrasted samples tolerate.

Imaging under low dose conditions

Observation of frozen hydrated samples in the electron beam needs a specialised imaging procedure called Low Dose Imaging. For this the searching, focussing and imaging tasks are done at different magnifications and different areas of the sample grid. Searching for a suitable imaging position is done at low magnifications of around $\times 1900$, focussing is done at the imaging magnification, but with the beam shifted away from the actual imaging area. For the imaging the beam is shifted back and a photo is recorded so as not to exceed the above mentioned electron dose.



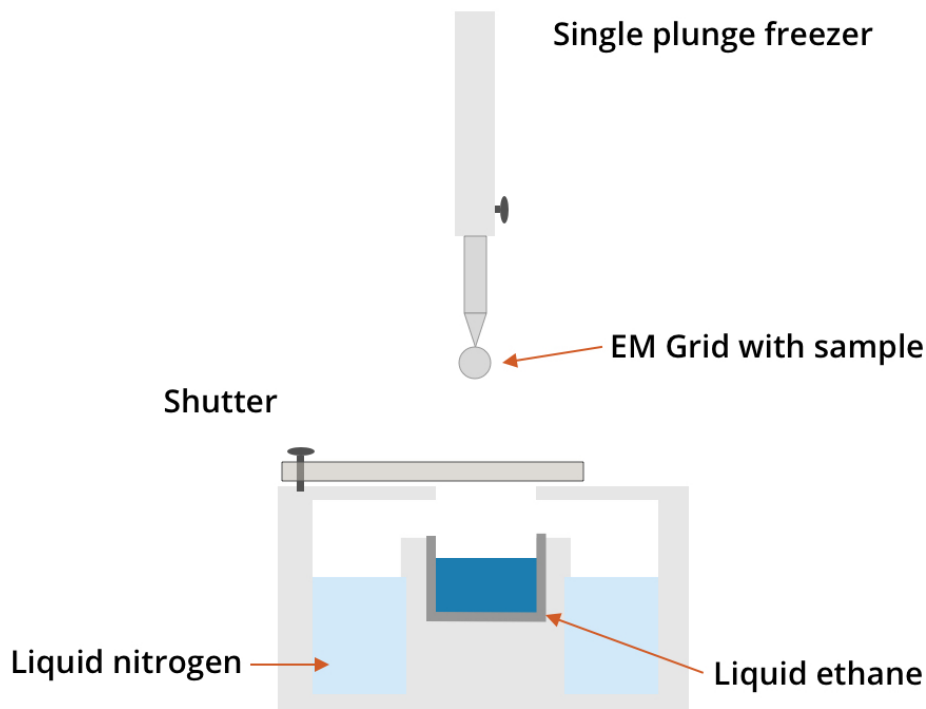


Real cryo sample with beam damage from focussing

The freezing process

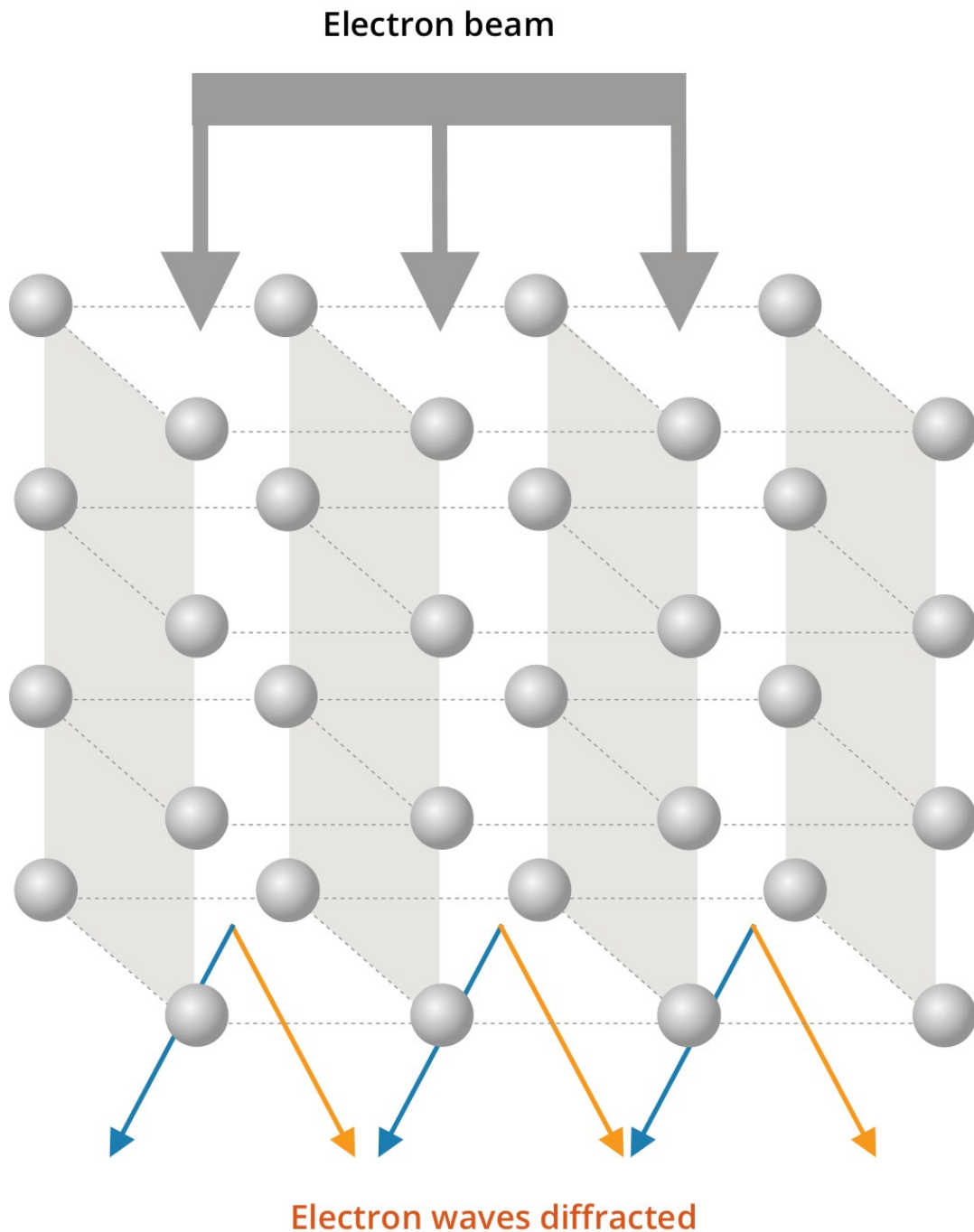
A plunge freezer is used to snap freeze the sample. In its simplest form this is a guillotine-like set-up that holds an EM sample grid with a pair of tweezers over a container with liquid ethane as a cryogen. The cryogen is kept at -180°C by liquid nitrogen.

The grid is covered with a thin carbon film, that usually has a pattern of tiny holes of about $1\text{-}2\mu\text{m}$ in it. Just before freezing, a small drop of the sample is placed on the grid and the excess liquid is blotted away with a piece of filter paper until only a thin water film of less than 100nm is spanning the holes. Just before the thin water film over the holes ruptures the plunge mechanism is triggered and the sample grid is plunged into the cryogen. The water is instantly frozen with the small protein particles randomly orientated and embedded in the ice. The rapid freezing rate of approximately $10,000^{\circ}\text{C/s}$ forms amorphous ice without any ice crystals.



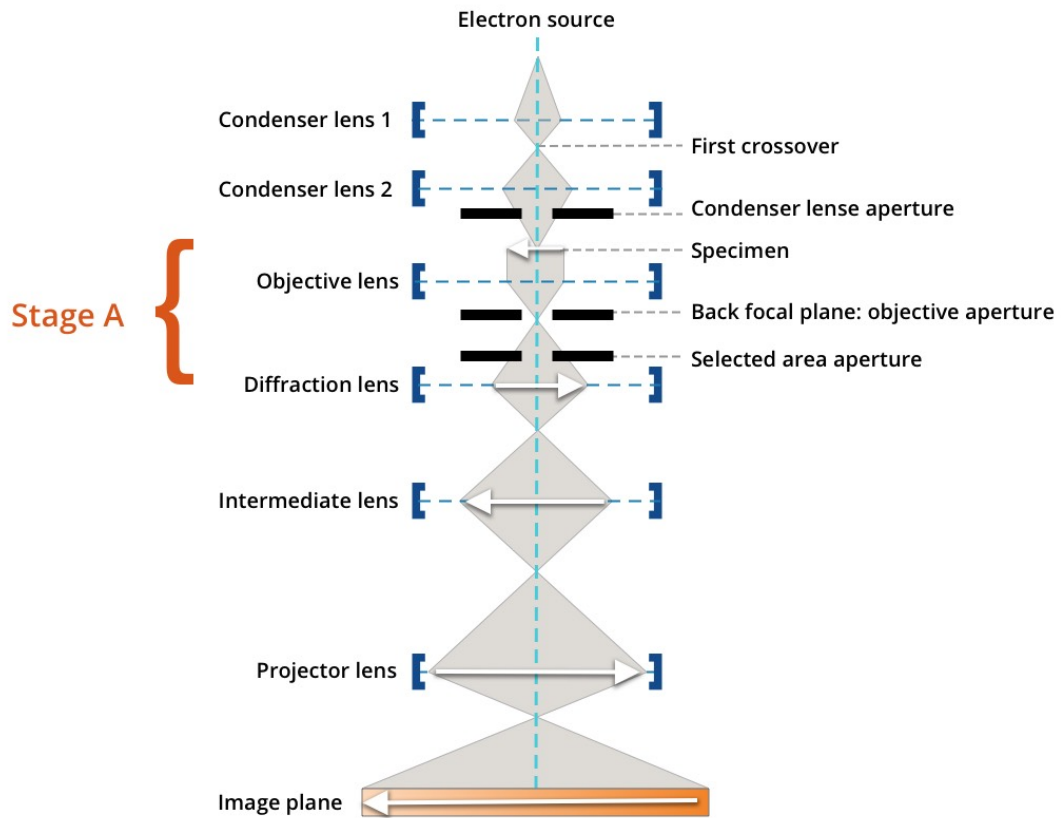
Diffraction - Introduction to diffraction

The TEM can provide information about the ultrastructure of a sample, including its crystalline nature. Crystal lattices act as a diffraction grating with interference patterns produced in the electron beam as it travels out from the lattice. These can be projected as an image of regular dots or rings.



Although not immediately obvious from bright field TEM micrographs, all crystalline specimens diffract the electron beam during TEM imaging, and it is largely this diffraction that creates the images we see. TEM diffraction and imaging are therefore closely linked. Let's revisit TEM image formation in more detail to help us understand how diffraction images are formed.

TEM images are formed in two stages. In the first stage, Stage A, the specimen scatters the electron beam. This scattered beam passes through an objective lens, which focuses it to form the primary image.



The second stage, Stage B uses the primary image obtained in stage A and magnifies this image using additional lenses to form a highly magnified final image.

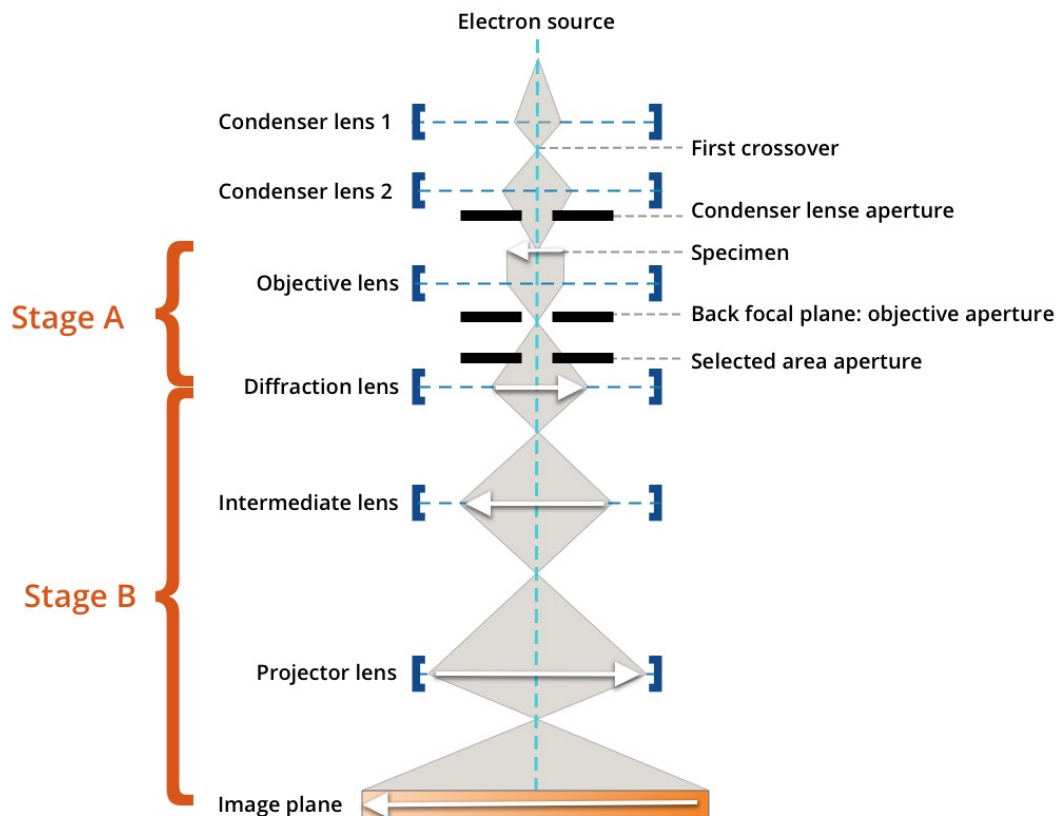
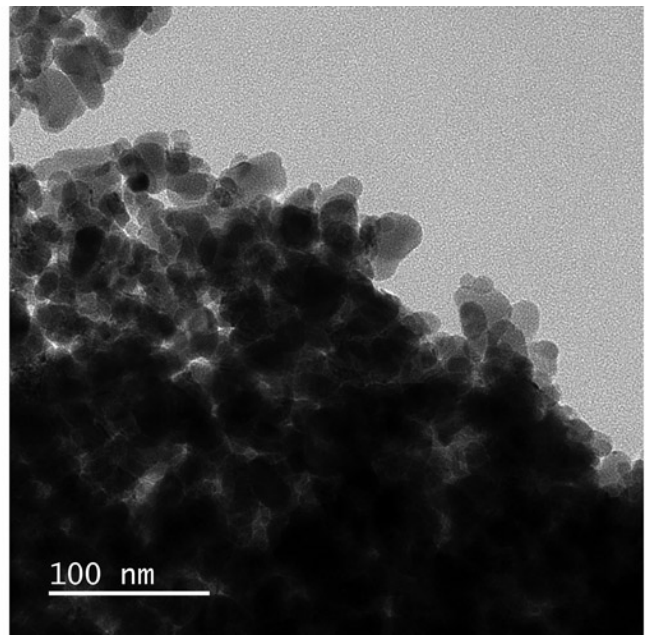
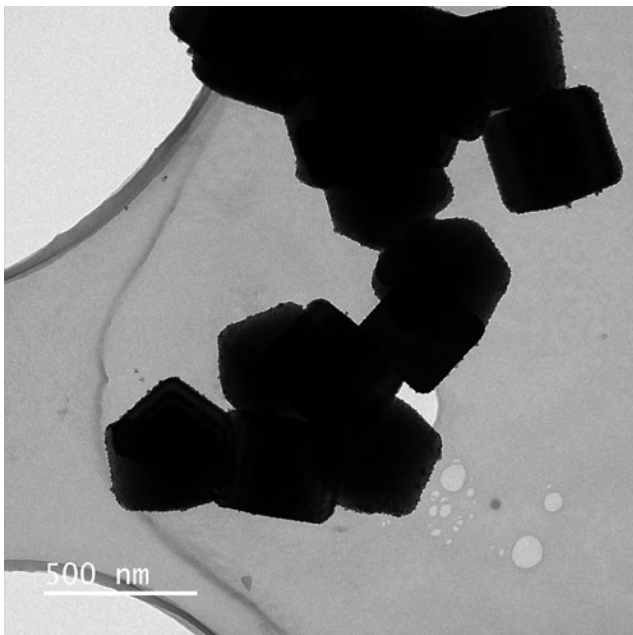


Image appearance

At high magnification, crystals may exhibit diffraction contrast. This can occur for one of two reasons:

1. Strongly diffracting regions of crystals can appear darker because there are fewer electrons transmitted along the primary beam. These crystals may be sitting on the grid holder at a tilted angle so that a lattice of the crystal lines up parallel to the beam.
2. Thicker regions of a sample can also appear darker due to greater scattering. This can be seen in the image here, where some crystals are lying on top of one another.

As the sample is tilted it will change in appearance to darker or lighter contrast depending on how the beam is interacting with the internal lattice. When strong diffraction conditions are achieved, the image will appear darker as more electrons are scattered outside of the objective aperture.



Two examples of poor TEM specimen preparation showing insufficient electron transmission due to the thickness of the particles in the specimen.

The diffracted beam

When the electron beam passes through a thin crystalline sample, it is diffracted by the atomic planes in the sample when the Bragg condition is satisfied. These waves interact constructively and are brought to focus at the back focal plane of the objective lens (see Planes) to form the diffraction pattern.

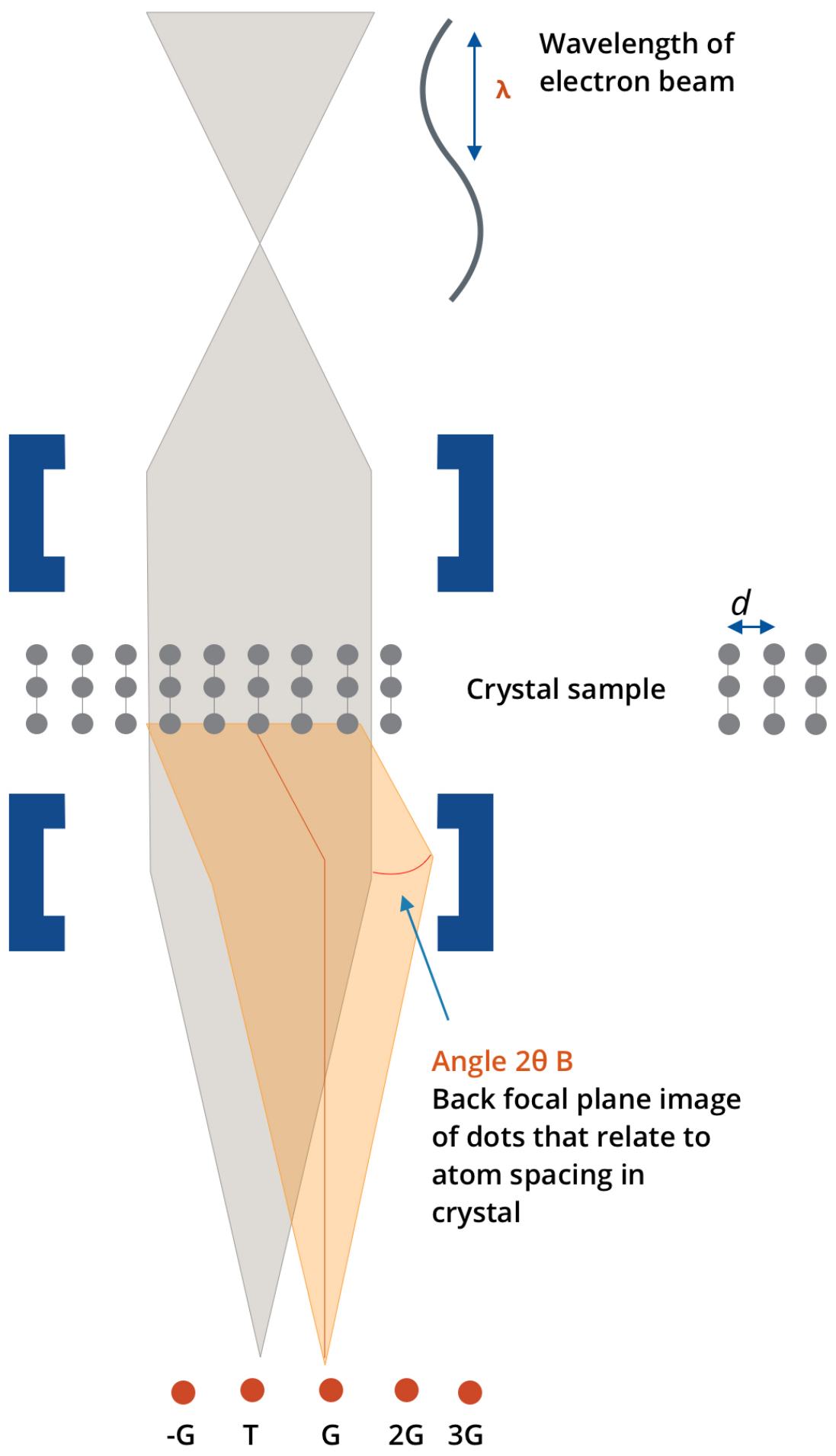
Unscattered electrons continue through to O to produce a central spot. The beam diffracted by angle $2\theta_B$ produces a spot, marked G. The distance between a diffracted (G) and transmitted (O) spot is inversely proportional to the corresponding lattice spacing in the sample.

The beam deflection angle and electron beam wavelength are important. Bragg's law describes the interaction:

$$\lambda = 2d \sin \theta_B$$

This equation can be used as long as the wavelength is less than the crystal interplanar spacing (d). This works for a TEM where the accelerated electron beam describes a wavelength of a few picometres. This means that for most crystalline materials the Bragg angle is much less than 1° .

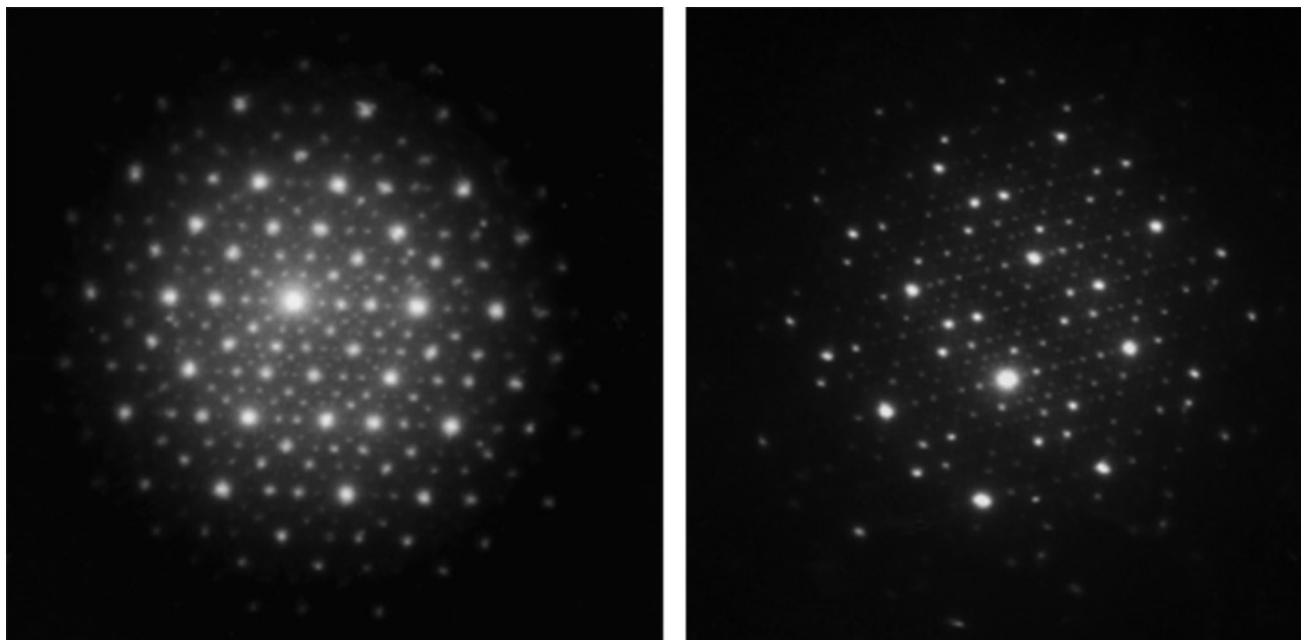
It is also important to know the camera length (projection distance) in order to calculate details about the sample. It can be set when photographing a diffraction pattern.



Tilting

The appearance of a diffraction pattern will depend on the orientation of the specimen relative to the electron beam. If the specimen is tilted so a plane of atoms or crystallographic direction satisfies the Bragg condition, distinctive diffraction patterns will be obtained with diffraction maxima (i.e. spots - often called reflections) in arrangements that reflect the crystal structure of the specimen.

To achieve this, samples in a TEM can be tilted. There are both single-tilt and double-tilt specimen holders. A double-tilt holder allows the user to tilt the specimen in two axes (X and Y). It is common to try and tilt a sample so that a crystal zone axis is parallel to the electron beam, and an example from a magnesium alloy is shown below. You can practice tilting a sample to a major zone axis using the TEM simulator.



Selected area diffraction pattern of two different zone axes within the same crystal. The different zone axes are obtained by tilting of the specimen. Images courtesy of Julie Cairney, University of Sydney.

Camera length

In order to calculate the lattice spacing in our sample we need to know the 'camera length'. Camera length is the distance from the sample to the projected image.

The diffraction pattern (formed in the back focal plane) forms the projected image on the screen and can be recorded, hence the term 'camera length' since this is where the camera is positioned. The projection process enlarges the distance between the reflections in the diffraction pattern.

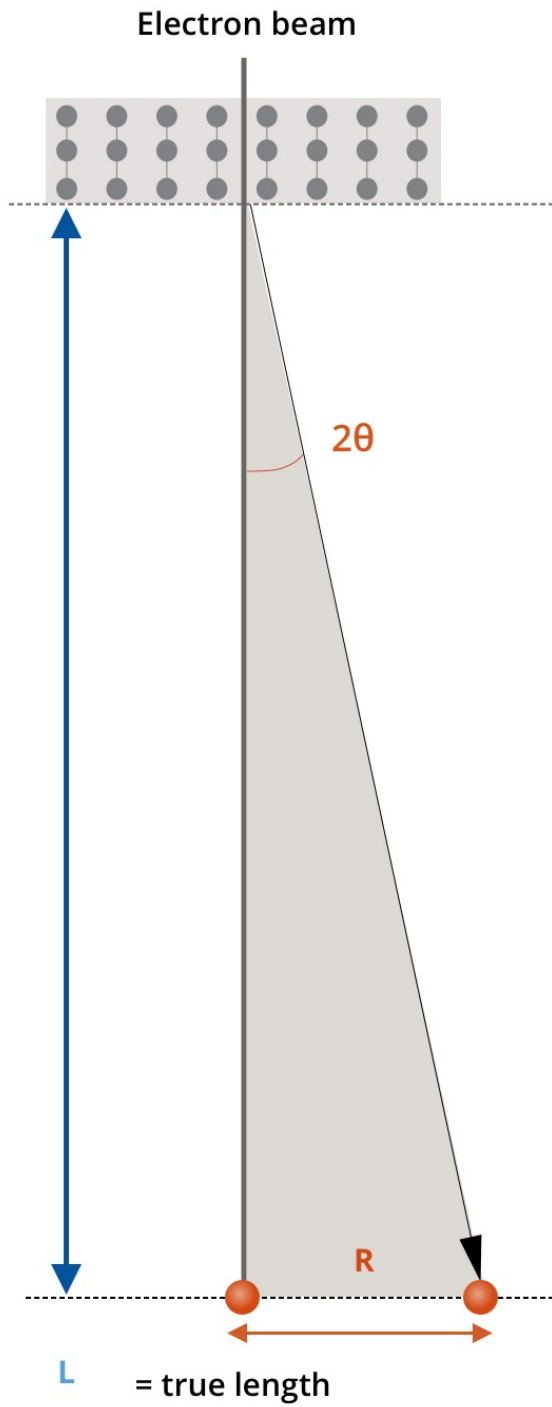
To obtain precise measurements from a diffraction pattern using a TEM, one must know the precise camera length.

A small camera length provides a pattern with little space between the reflections and a large camera length provides a pattern with large spaces between the reflections. So, too small a length and the diffraction image only fills up a small region of the projection screen. Too large and part of the diffraction pattern can be lost at the edges beyond the projection screen.

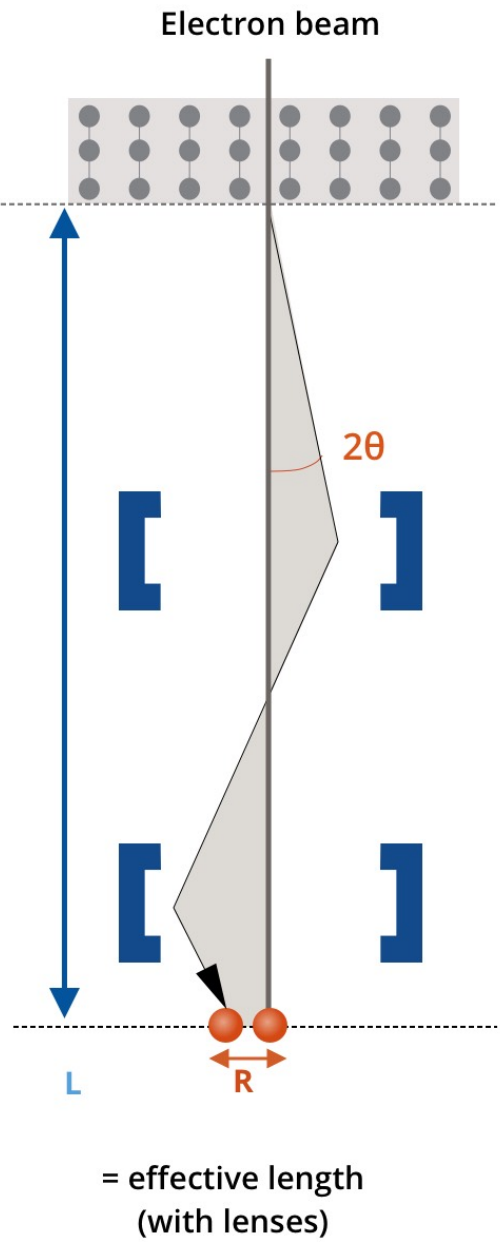
It must be remembered that the camera length must be calibrated for accurate measurements. This can be done by using a calibration standard sample for which the lattice spacing is known.

Once an accurately calibrated diffraction pattern is achieved, the information in the pattern is used to determine lattice planes and in the indexation of diffraction patterns.

True length



Effective length



$$L = \frac{R}{2\theta}$$

Kikuchi patterns

Bragg scattering is diffraction of inelastically scattered electrons and can lead to the formation of pairs of parallel lines in the diffraction pattern called Kikuchi lines. For each plane of atoms in the sample there exists a pair of parallel lines, rather like train lines. The various sets of Kikuchi lines intersect in diffraction space in a manner that represents the arrangement of crystal planes in real space. This is called a Kikuchi map. These lines can help an operator to tilt a crystal around to find different crystal planes. Kikuchi Patterns are a useful phenomenon to use when initially learning how to tilt crystals because they form regular intersecting lines over a zone axis.

This spot diffraction pattern shows Kikuchi lines on the left side of the pattern. This pattern is in the process of being tilted and not yet on the zone axis.

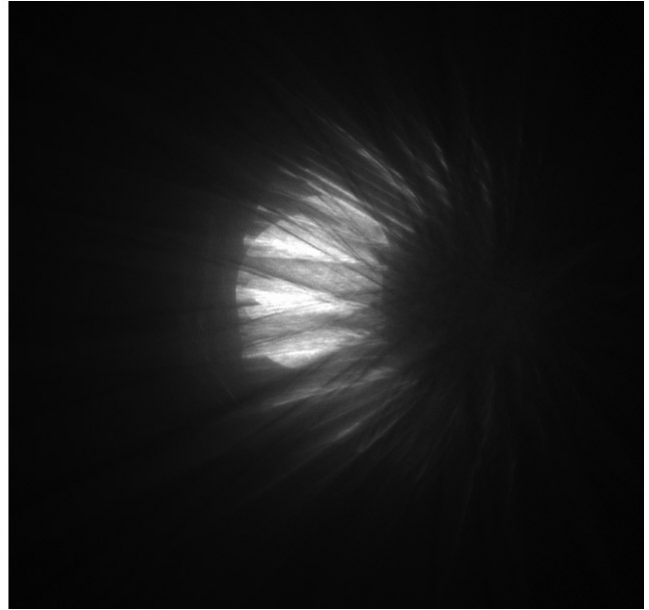
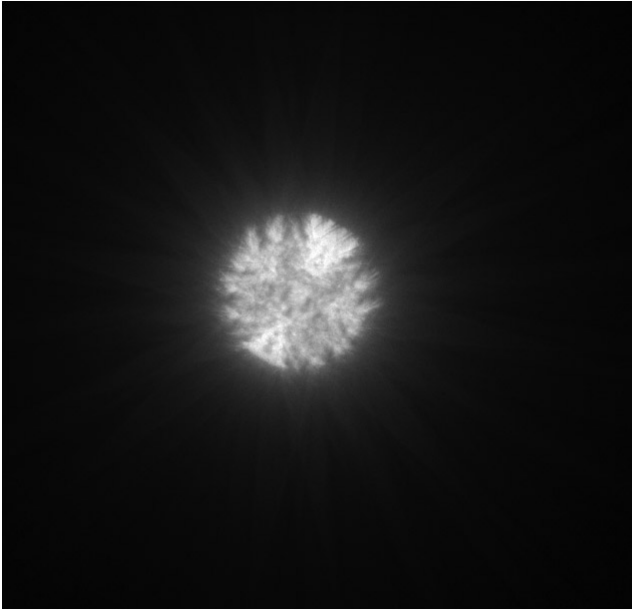
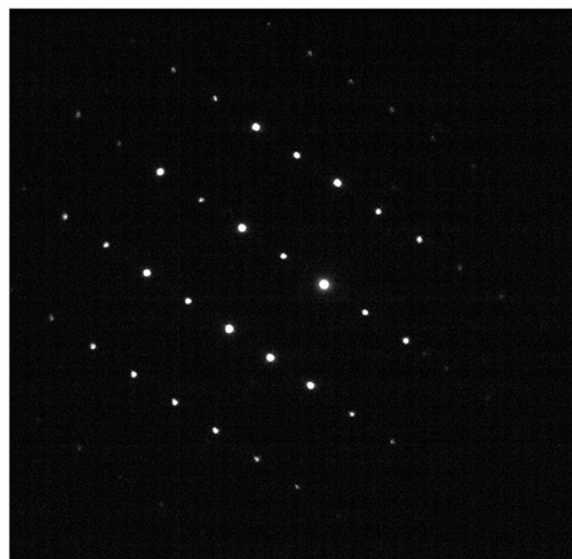
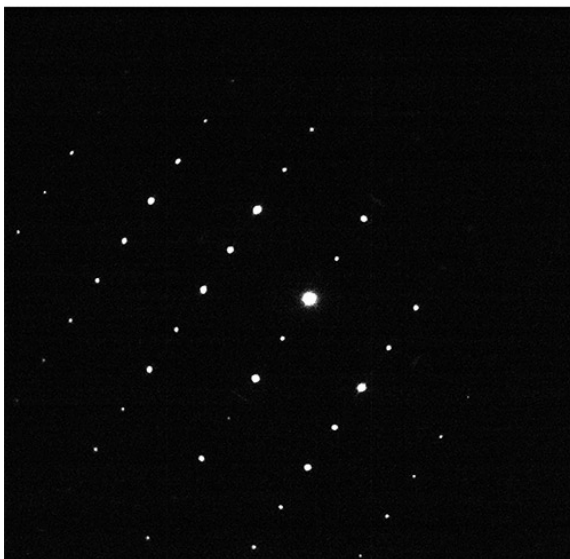
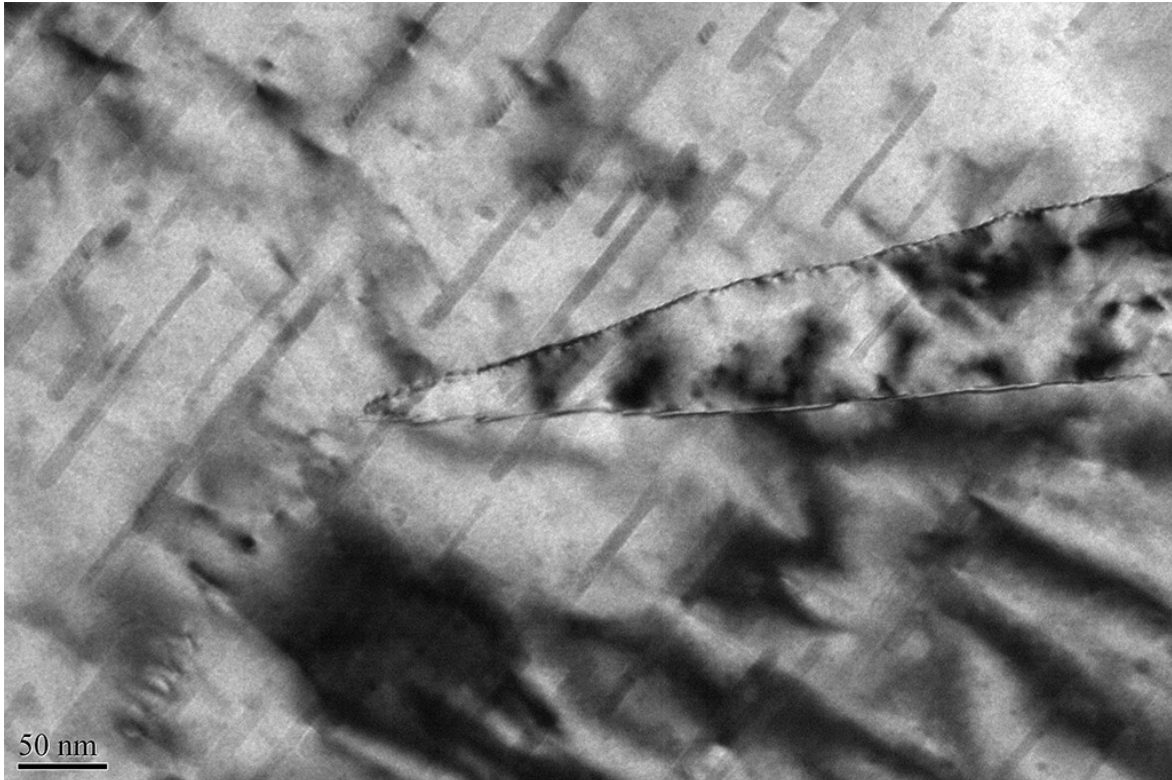


Image shows Kikuchi pattern obtained by convergent beam diffraction. Kikuchi diffraction pattern on the left is on a major zone axis. Kikuchi diffraction pattern on the right shows specimen off-axis. Image courtesy Ashley Slattery, Adelaide Microscopy.

Selected area diffraction (SAD)

Often, in diffraction mode, it is necessary to isolate a local region so that only this region produces a diffraction pattern. This is achieved by introducing the selected area diffraction (SAD) aperture into the column. When the selected area diffraction aperture is used to limit the area that is used for obtaining a diffraction pattern, this is called selected area diffraction (SAD).

When the sample is oriented with a zone axis pattern parallel to the electron beam, the beam-sample interactions generate the diffraction pattern in the back focal plane of the objective lens as a regular array of reflections. This is seen projected onto the viewing screen as an array of reflections organised in a predictable manner based on the crystal structure of the sample.



Example of selected area diffraction in a magnesium alloy. The deformation twin shown in the micrograph has a common crystallographic rotation axis with the parent. Both the image and the SAD are shown parallel to this common axis. The orientation of the parent is shown in the SAD on the left, and the orientation of the twin shown in the SAD on the right. Image courtesy Nikki Stanford, University of South Australia

Ring patterns

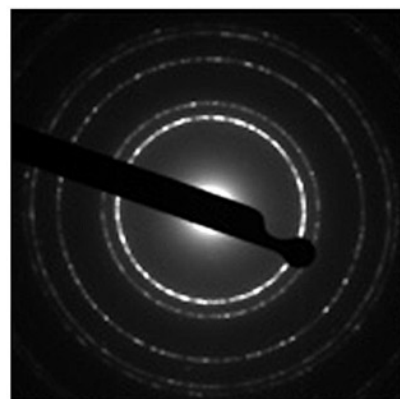
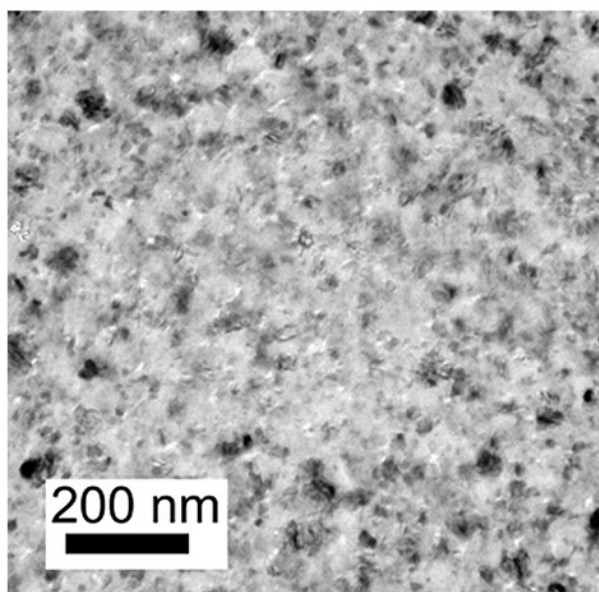
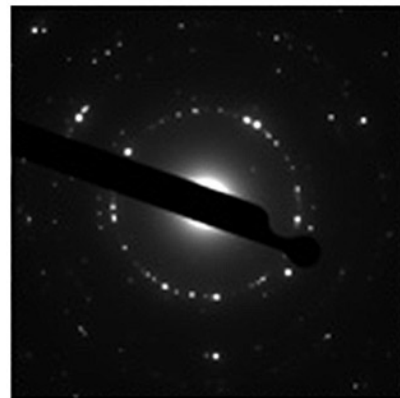
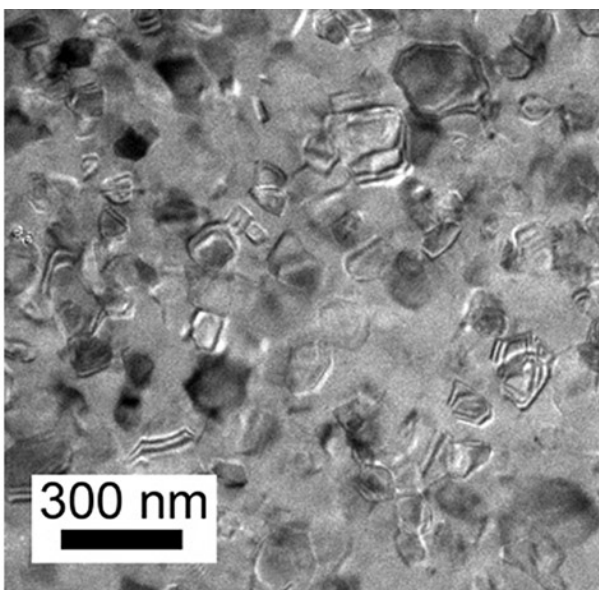
Sometimes, instead of intensity spots, the electron diffraction pattern is composed of concentric rings.

Materials that contain no long-range order in the atomic lattice produce diffuse ring diffraction patterns with no discrete reflections and one or possibly two diffuse rings of maximum intensity. Amorphous samples, e.g. polymers and metallic glasses, produce this kind of pattern.

Some thin films used as substrates to hold samples are made of materials such as amorphous SiN. This is the ring pattern from such a film. The bar across the pattern is the "beam stop" used to cover the bright central beam spot so that the more diffuse rings can be captured as a digital image.

If the material is a collection of a large number of crystals, with different orientations, then individual reflections are seen within the rings.

This image is a selected area diffraction (SAD) pattern from a Zr/Ni/Cu-based alloy and zirconia.

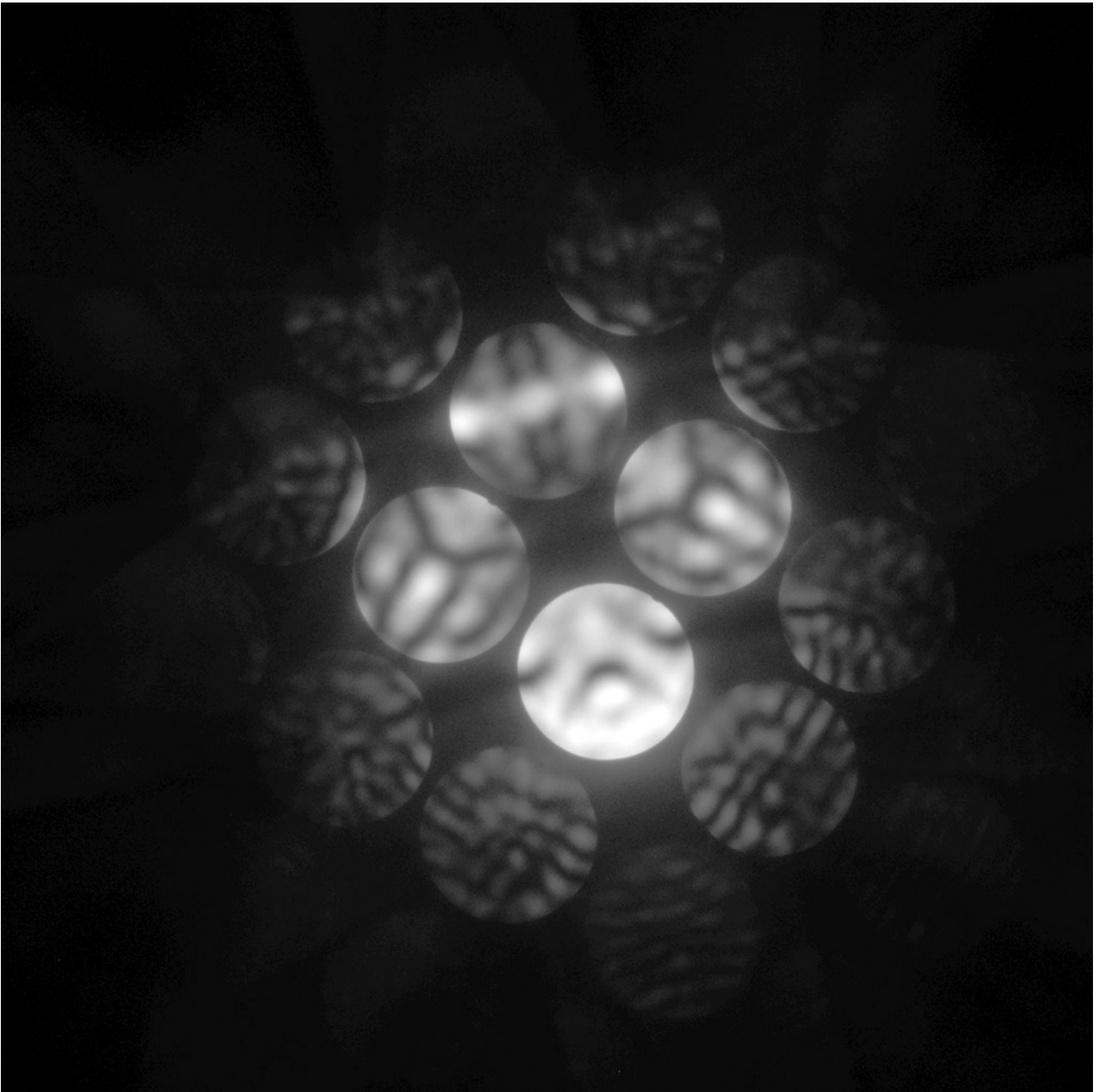


Diffraction patterns from fine grained specimens. Upper image shows individual diffraction spots from each grain in the selected area. Lower image shows nano-sized grains, diffraction from so many crystals forms full rings in the diffraction pattern. Image courtesy Julie Cairney, University of Sydney

Convergent beam electron diffraction (CBED)

When the electron beam is converged on the sample to a point (method = convergent beam), instead of using a parallel stream of electrons through the sample, the diffraction pattern forms discs instead of spots in the back focal plane of the objective lens.

These discs can contain detail that provides information about the crystal structure of the specimen. In these images we see almost perfect mirror symmetry in the patterns within the discs. These symmetries can be used to determine the point and space group of the crystal.



An example of a convergent beam electron diffraction (CBED) pattern from an Al-Cu alloy. Image courtesy Xiaofen Tan, Laure Bourgeois and Phillip Nakashima, Monash Centre for Electron Microscopy

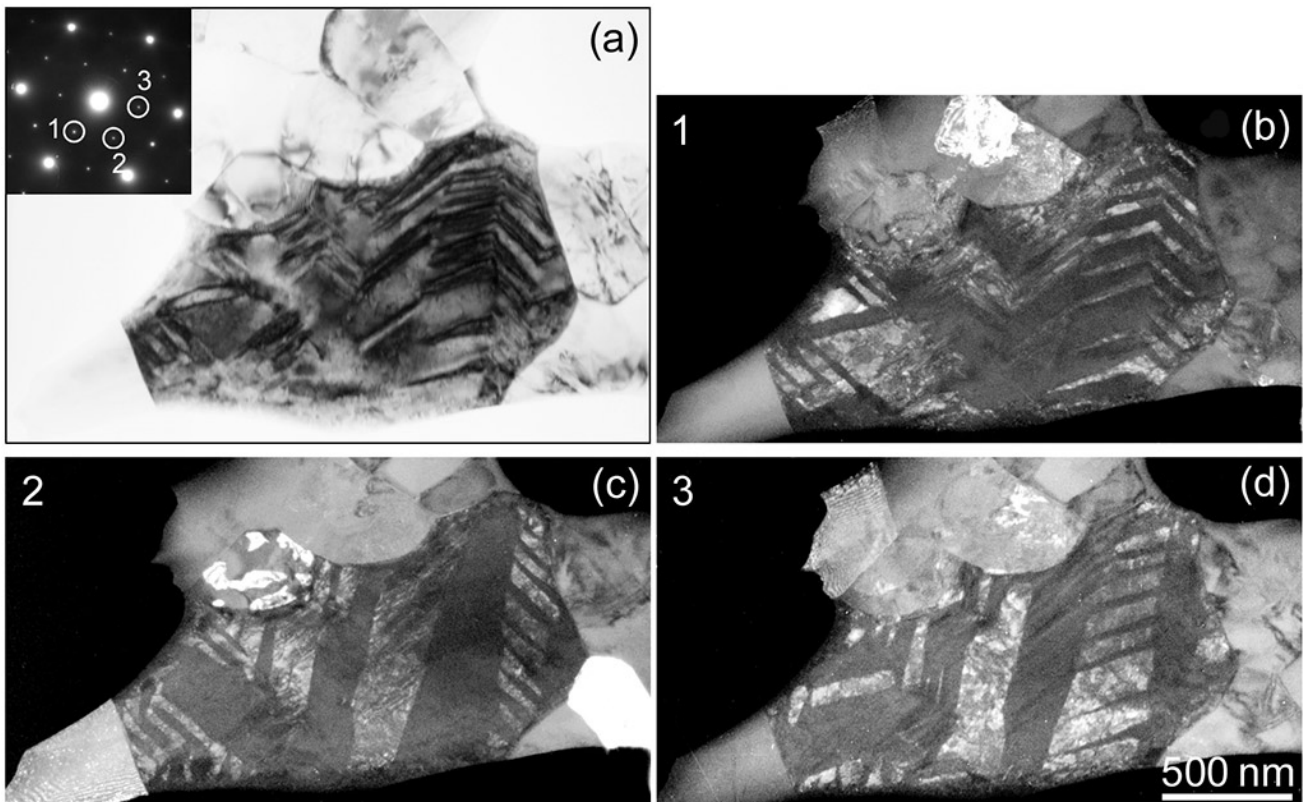
Dark-field imaging

Dark-field images are produced by collecting only the scattered electrons. The primary aperture is used to exclude the unscattered beam from being collected below the sample. Dark-field imaging is achieved by blocking the unscattered beam and only allowing scattered electrons to form the image.

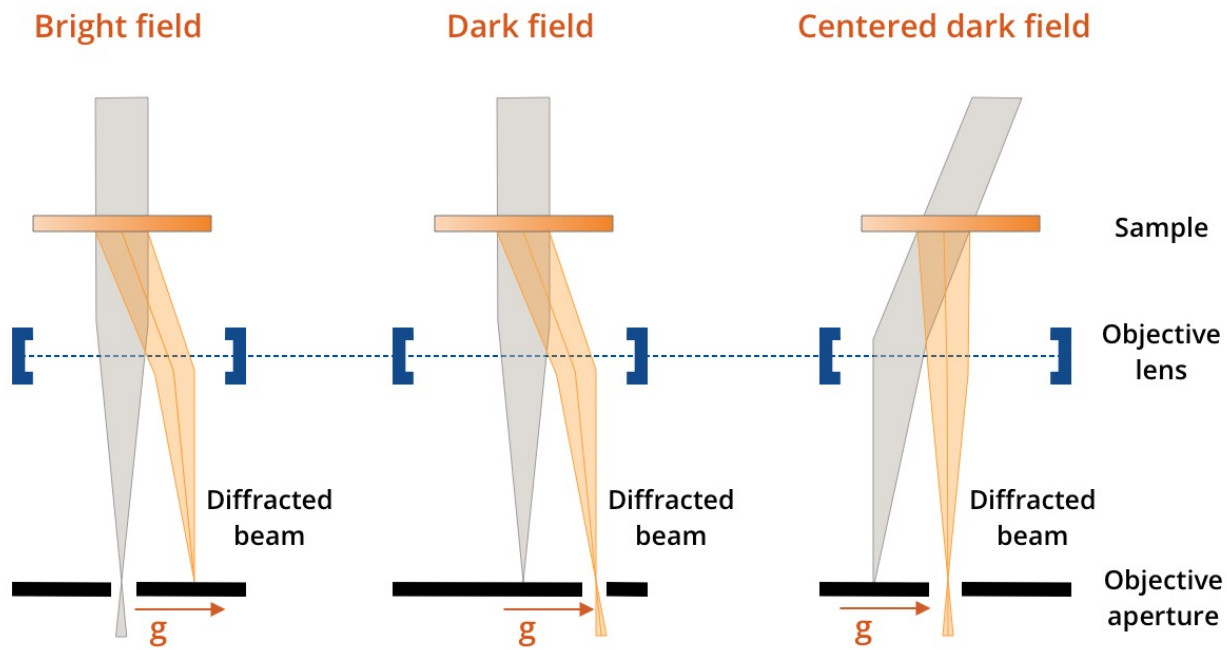
There are a few ways this can be achieved. Methods 1 and 2 involve putting the objective area into to column.

Method 1: (Dark-field) is where an objective aperture is introduced and moved so that it excludes the unscattered beam but allows some signal to pass through from a specific area of interest in the diffraction pattern, for example a specific intensity spot.

Method 2: (Centred Dark-field) is where the beam is tilted so that the unscattered beam path is blocked by the objective aperture. In this way a particular intensity spot in a diffraction pattern can be centred as the new 'main beam'. The dark-field image will be produced only from those electrons being diffracted along this axis. The image is then focussed in the sample plane (on the sample). Regions where no scattering occurs, such as where the primary electron beam passes straight through the sample, appear black (e.g. in areas around the sample). This kind of imaging is useful in studying crystal defects, and for the imaging of specific crystallographic phases.

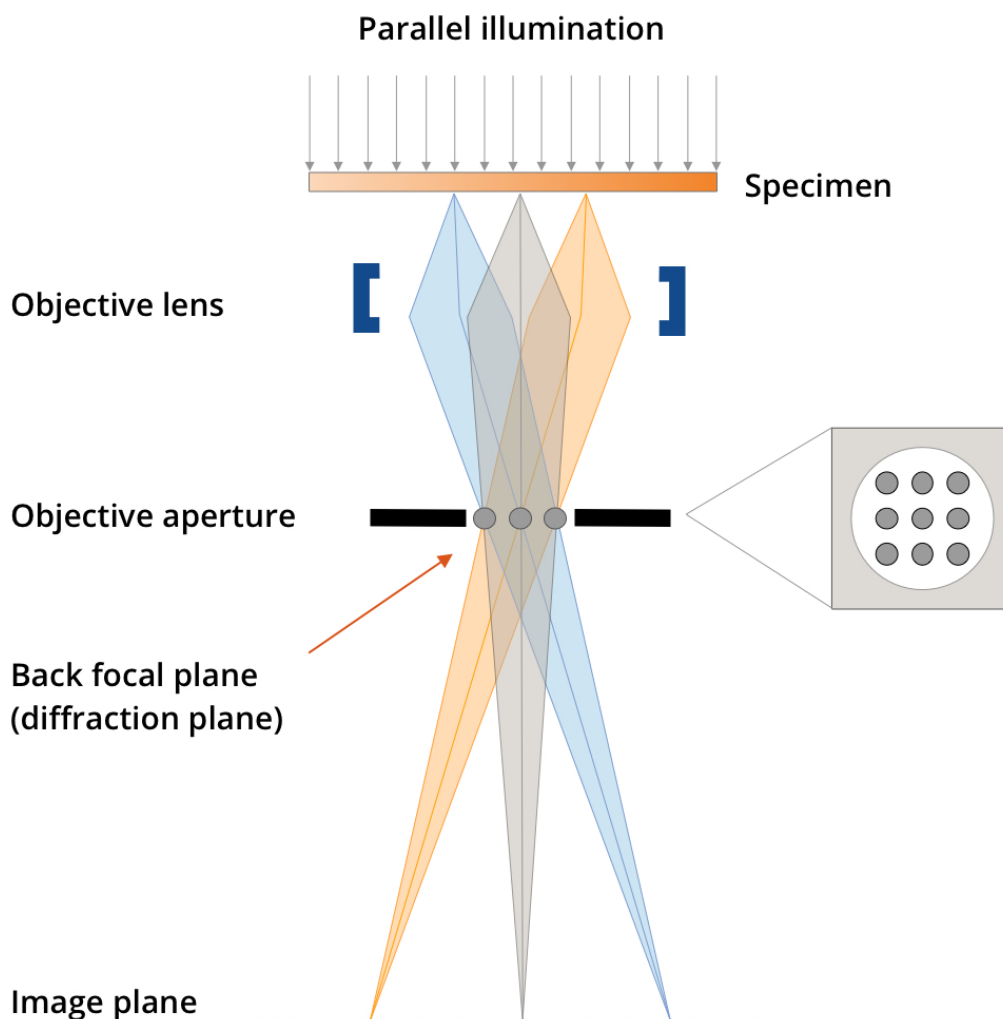


Imaging of zirconia. (a) is a bright-field image and (b-d) are dark-field images produced by selecting one of the independent tetragonal reflections denoted by circles in the inset of (a). This technique reveals the 'herringbone' structure within this specimen. Image courtesy Julie Cairney, University of Sydney.



High resolution imaging - What is high resolution imaging?

In contrast to bright-field or dark-field images, which typically use one transmitted or diffracted beam; high resolution imaging is used to form images using multiple beams. In such conditions contrast is generated by changes in phase. For this reason HRTEM is sometimes termed phase-contrast imaging. Changes in phase between the transmitted and diffracted beams result in constructive and destructive interference in the front focal plane of the objective lens. This is known as the exit wavefunction. This exit wave is then focused by the objective lens, which introduces further phase changes depending on the defocus and other lens aberrations, mainly spherical aberration. Typically a Scherzer defocus of approx. -60 nm is used (though this will vary with each microscope). At this defocus the phase change caused by aberrations in the objective lens are minimised. It is important to note that direct assessment of contrast in HRTEM is not possible without rigorous computer simulations and is typically only possible for thin sections <50 nm.



A simplified diagram showing the formation of a high resolution image of a crystalline thin specimen in TEM. An objective aperture (OA) is inserted in the back focal plane to allow the central spot together with its nearby spots to go through to form an image. The right panel displays a top view of the centered OA surrounding the undiffracted beam and the first g-vectors.

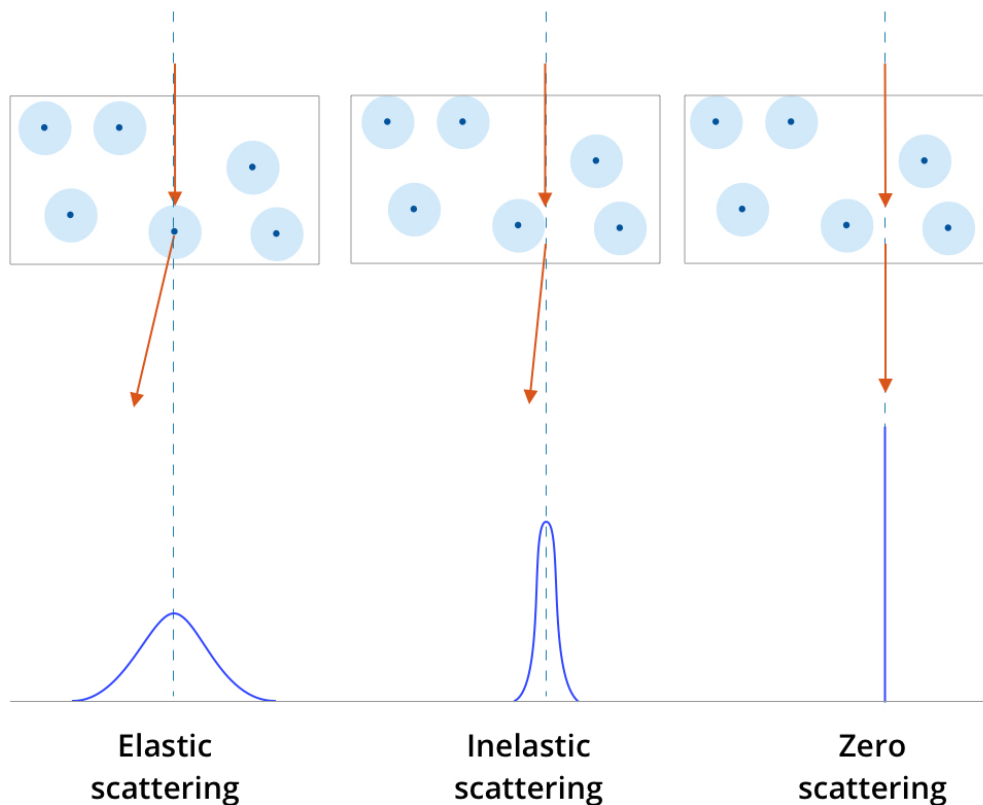
Scanning TEM (STEM) - Bright field STEM

Scanning transmission electron microscopy (STEM) involves focusing an electron beam into a small probe and scanning it across a sample (similar to a SEM). The image is built up pixel-by-pixel by collecting the electrons transmitted through the sample at each point in the scan. The sample requirements are similar to those for conventional TEM analysis, as sufficient electrons must be transmitted through the sample to build up the image. The resolution of the image depends on the probe size, with atomic resolution possible on high-resolution instruments. As in the SEM, the magnification of the STEM image is simply the ratio between the size of the micrograph formed and the size of the area rastered by the electron beam.

Beam-sample interactions

When the probe scans across a thin crystalline specimen, the transmitted electrons undergo different interactions and consequently emerge at various angles. There are three typical interactions of the electrons with the specimen:

1. Electrons transmitted through the specimen pass between the atoms without being scattered.
2. Electrons hit the electron cloud of an atom and lose energy by knocking the electrons out of their shells.
3. Electrons travel close to the nucleus of the atom, where the Coulomb field of the nucleus scatters the incident electron into high angles. The scattering generated by the Coulomb field of the nucleus is considered to be an elastic scattering event and the power of scattering the incident electrons increases with the atomic number of the atom (Z). This property of scattering is utilised in STEM mode to examine specimens containing more than one type of atoms. It is particularly useful when there is a large atomic number contrast between the features of interest, for example, a copper precipitate in an aluminium matrix.



Use of ronchigrams in STEM

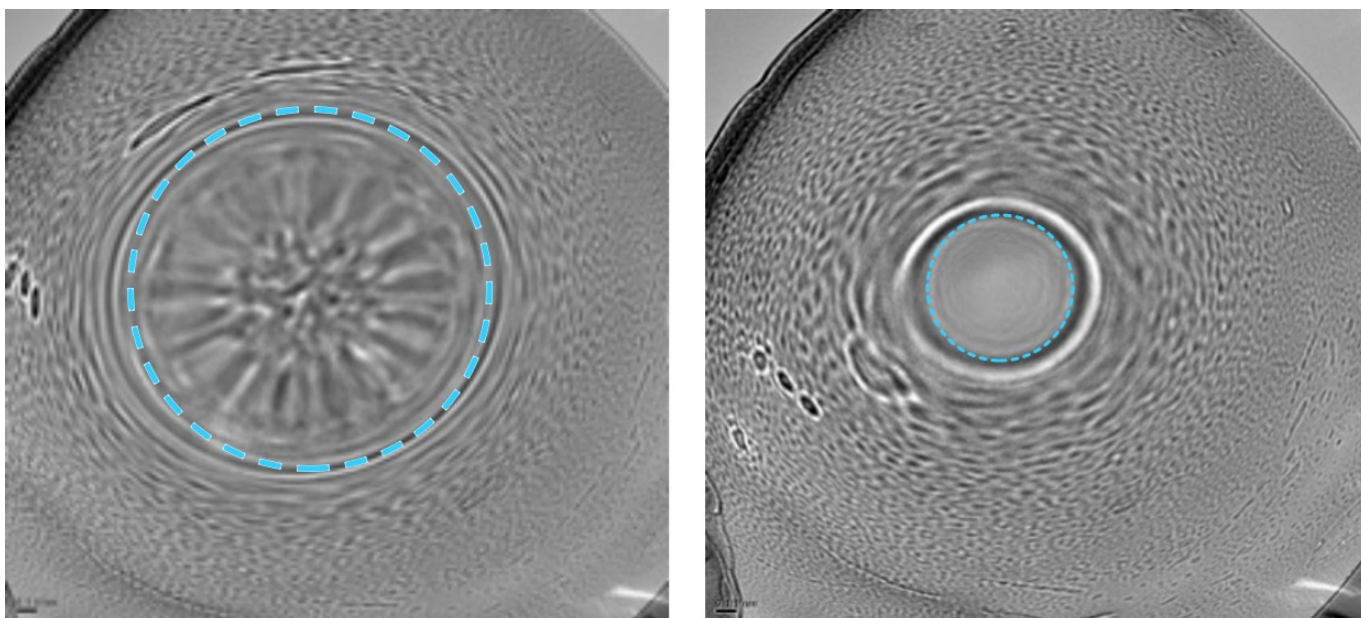
What is a ronchigram?

A ronchigram is a type of diffraction pattern. It's used to align the microscope in STEM mode to obtain the best STEM images possible from your microscope. STEM mode works a bit differently to normal bright field TEM imaging: whereas TEM involves a large unfocused beam of electrons interacting with the specimen, in STEM, a focused beam of electrons (or probe) is scanned across the specimen. For each position of the STEM probe on the specimen, a diffraction pattern is obtained – this is the ronchigram. It is these individual ronchigrams that are integrated by the software to form the final STEM image.

How and why we use ronchigrams?

Ronchigrams are very sensitive to the shape, size and position of the electron probe used to form STEM images. To get the best STEM images, the electron probe must be of the right shape and size and must be focused on the top of the specimen. This is why most microscope adjustments are made directly on the ronchigram.

A thin amorphous region is usually used for this to avoid complications caused by crystal structure. In the figure below you can see that there are two main regions in the ronchigram: a central circular region bounded by concentric rings of contrast, and the region outside of these rings. No part of this outer region should be used to optimise the STEM imaging as it contains distortions from the scattered beams.



Ronchigrams obtained from a thin amorphous carbon film in STEM mode.

The first adjustment is to focus the incident electron probe on the surface of the specimen, by changing the specimen height relative to the probe. As the specimen approaches the correct height, the ronchigram changes in appearance from having a speckled centre region, shown in the figure on the left, to having a clear, featureless central region, shown in the figure on the right.

Once the incident electron probe is focused on the specimen, the ronchigram can be used to correct for astigmatism, i.e. to make sure the probe is round. This is done by overlaying a perfect circle on the centre of the ronchigram and adjusting the stigmators to make the ronchigram round to match the circle. Finally, the ronchigram is used to centre the condenser aperture, by aligning it with the outer rings of the ronchigram. The size of the condenser aperture should match the central region of the ronchigram, as shown by the blue circle in the image on the right.

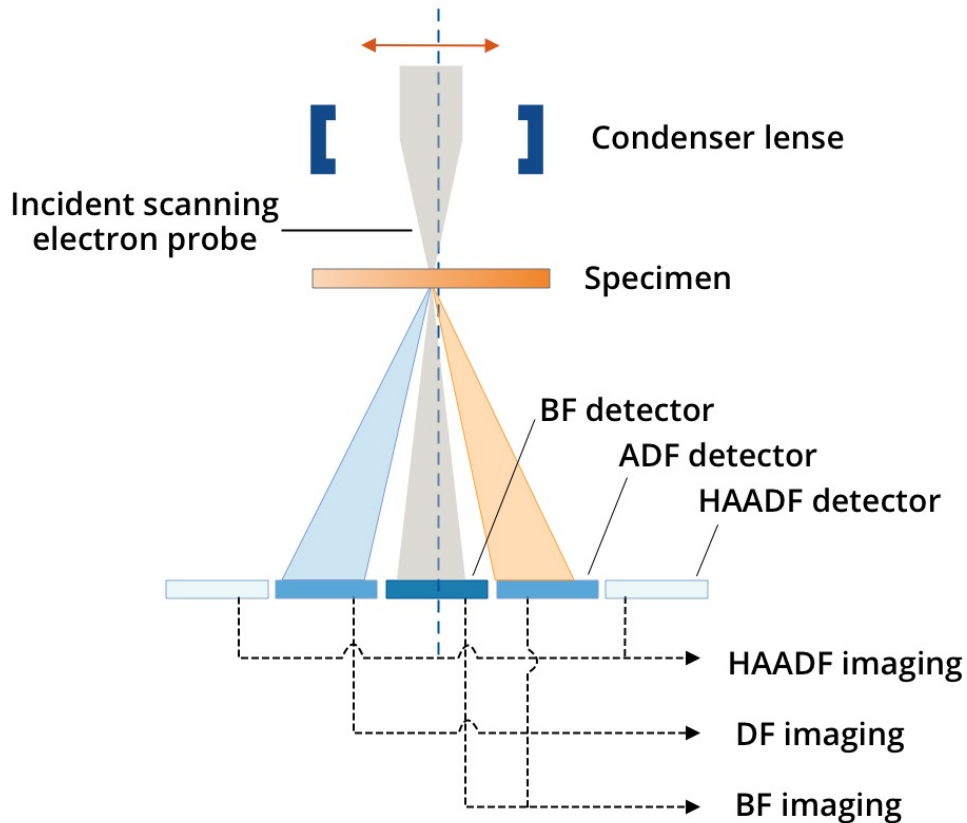
Once the condenser aperture has been inserted to select just the central part of the ronchigram, the incident probe can be scanned over the specimen and a STEM image obtained.

So, you can see from this introduction to the ronchigram that it is a vital tool for your STEM imaging!

STEM detectors

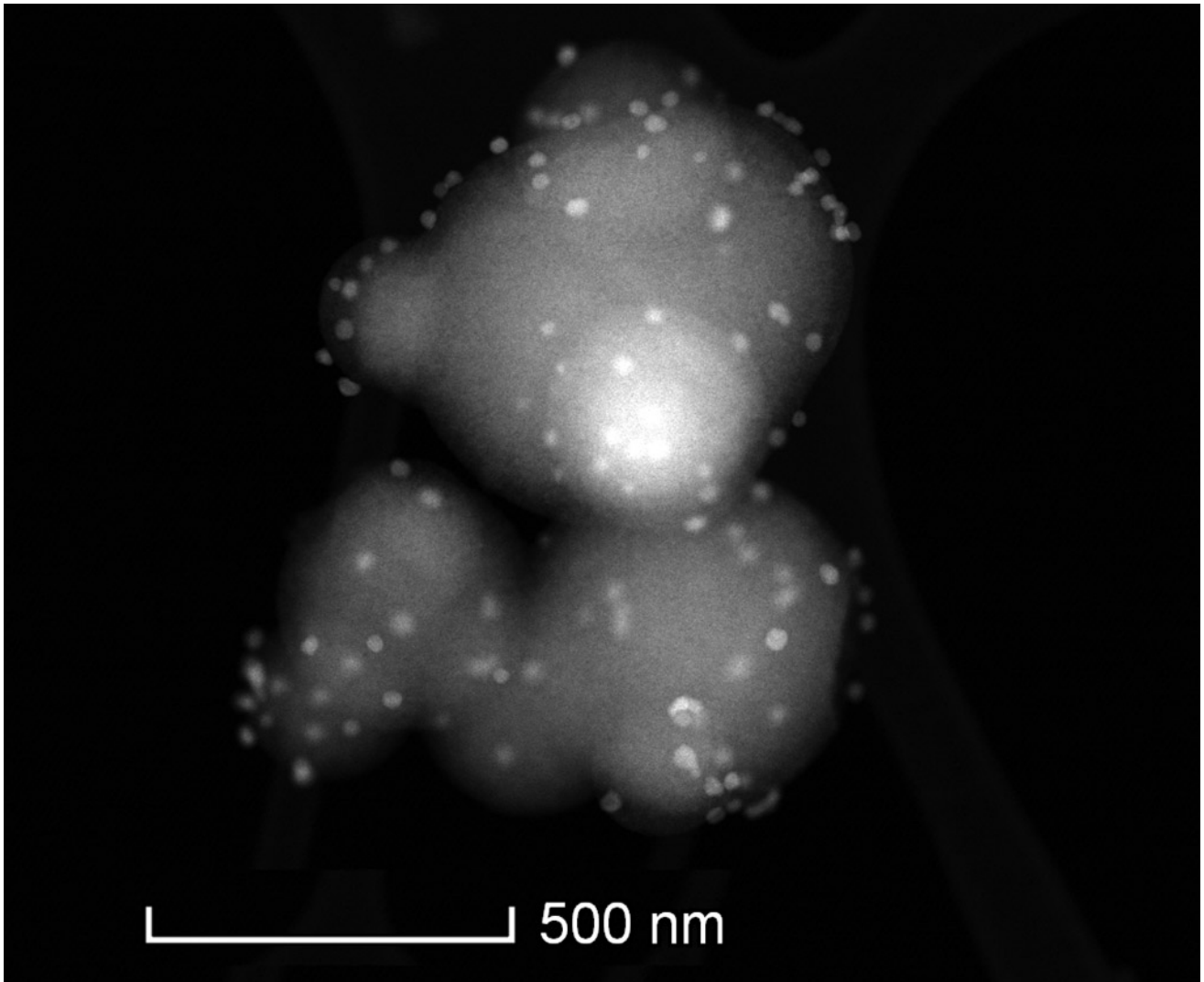
Several detectors are used in STEM mode to collect the electrons at different angles simultaneously, and the intensity is displayed on a screen as micrographs of the region being scanned.

- Bright field: The BF detector is a disc that collects all of the electrons directly transmitted on the optical axis of the microscope.
- Annular Dark-field: The ADF is an annular detector, concentric with the BF detector, allowing the BF signal to pass through while collecting the diffracted and/or scattered electrons.
- High-angle annular Dark-field: The HAADF detector is a large annular detector that collects electrons scattered at very high angles.



High-angle-annular dark-field (HAADF)

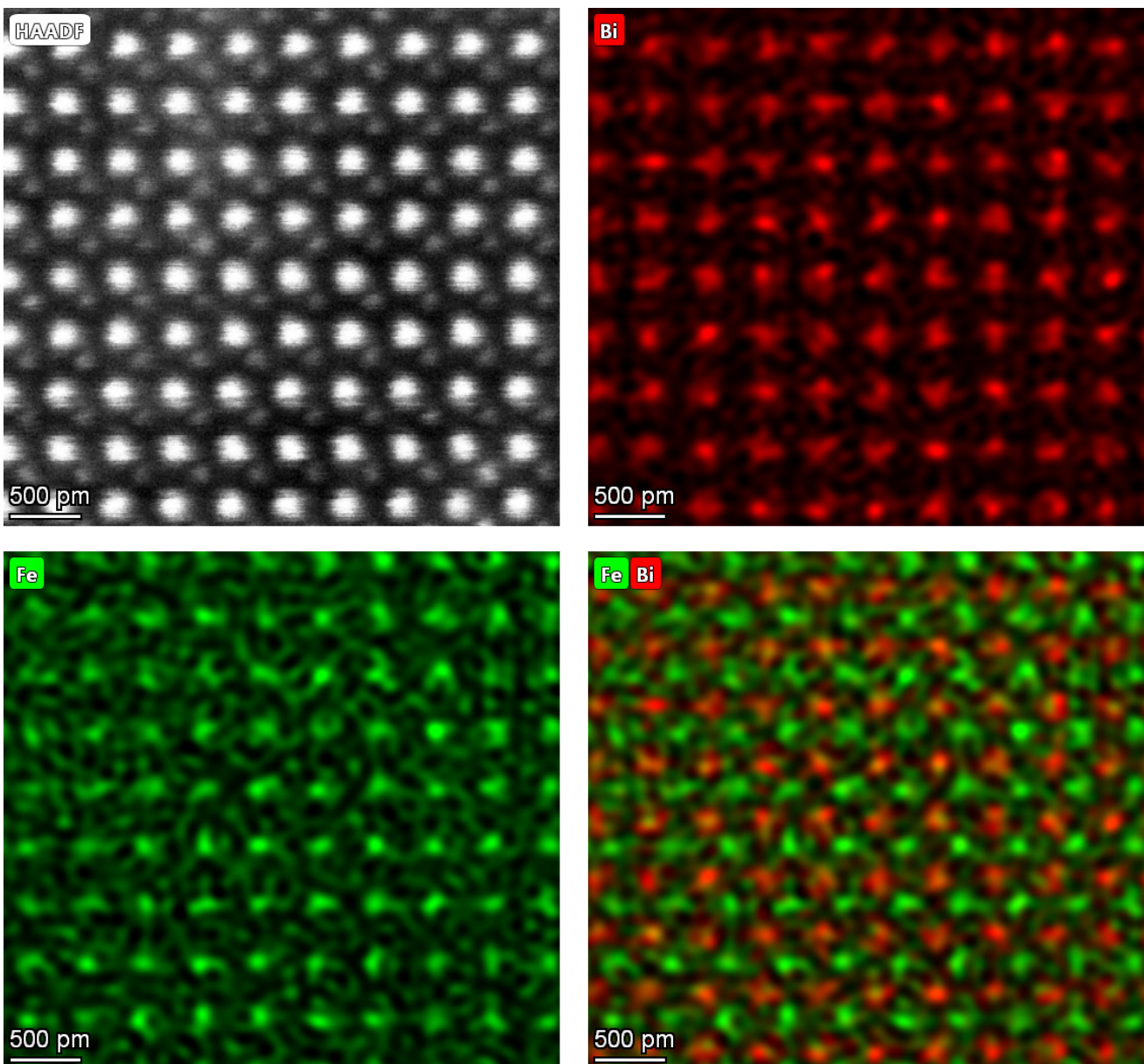
High-angle annular dark-field (HAADF) STEM, also referred to as Z-contrast imaging, involves collecting only those electrons scattered through very large angles. The resulting image shows mass- (or Z-) contrast with higher atomic number regions of the sample appearing brighter than light element regions.



HAADF STEM image of an SiO_2 particle coated with nickel nano-particles. Note the brighter colour of the nano-particles due to their higher atomic number compared to the SiO_2 . Image courtesy of Jiangtao Qu, University of Sydney

Energy dispersive spectroscopy (EDS) - Introduction to EDS

High energy electrons impacting on matter will cause the emission of X-rays, the energy of which is characteristic of the emitting atom. These can be measured using a technique called energy dispersive X-ray spectroscopy (EDS). Emitted X-ray signals provide compositional information on the specimen and originate only from the region illuminated by the beam. The beam can be broad, or it can be focused onto a region just a few nanometres in diameter. It is therefore possible to obtain compositional information with very high spatial resolution, e.g. from individual nanoparticles. By analysing the EDS spectrum, it is possible to obtain qualitative information (which elements are present) and semi-quantitative information (how much of each element is present) with an accuracy of perhaps a few percent. The performance of EDS is somewhat specimen dependent, but lower detection limits of about 0.5 wt% are typical. EDS techniques have quite poor sensitivity for very light elements (B, C, N, O). It is possible to determine that a material is an oxide or a carbide for example, but little more. Fortunately, the complimentary technique of electron energy loss spectroscopy (EELS) shows excellent sensitivity for light elements and between the two techniques a complete analysis is usually possible.



Energy dispersive spectroscopy imaging of the interface between BiFeO_3 and SiTiO_3 . Image courtesy of Jiangtao Qu, University of Sydney

Quantification of EDS data

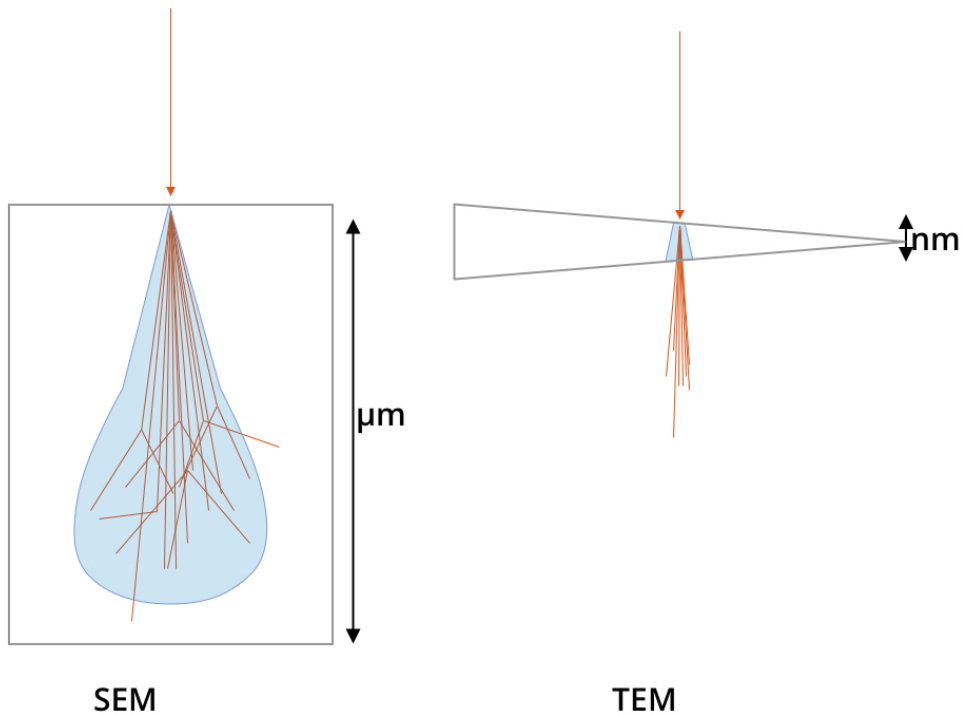
Unlike in the SEM, where quantification of EDS data is difficult, in the TEM the reduced interaction volume can simplify the quantification process.

The basis of quantification of EDS in TEM is to assume that the concentrations of elements C_A and C_B in the sample are related to the X-ray intensities (I_A and I_B) by:

$$\frac{C_A}{C_B} = k_{AB} \frac{I_A}{I_B}$$

Where k_{AB} is a sensitivity factor which varies according to each detector, microscope and accelerating voltage used and it is assumed that $C_A + C_B = 100\%$.

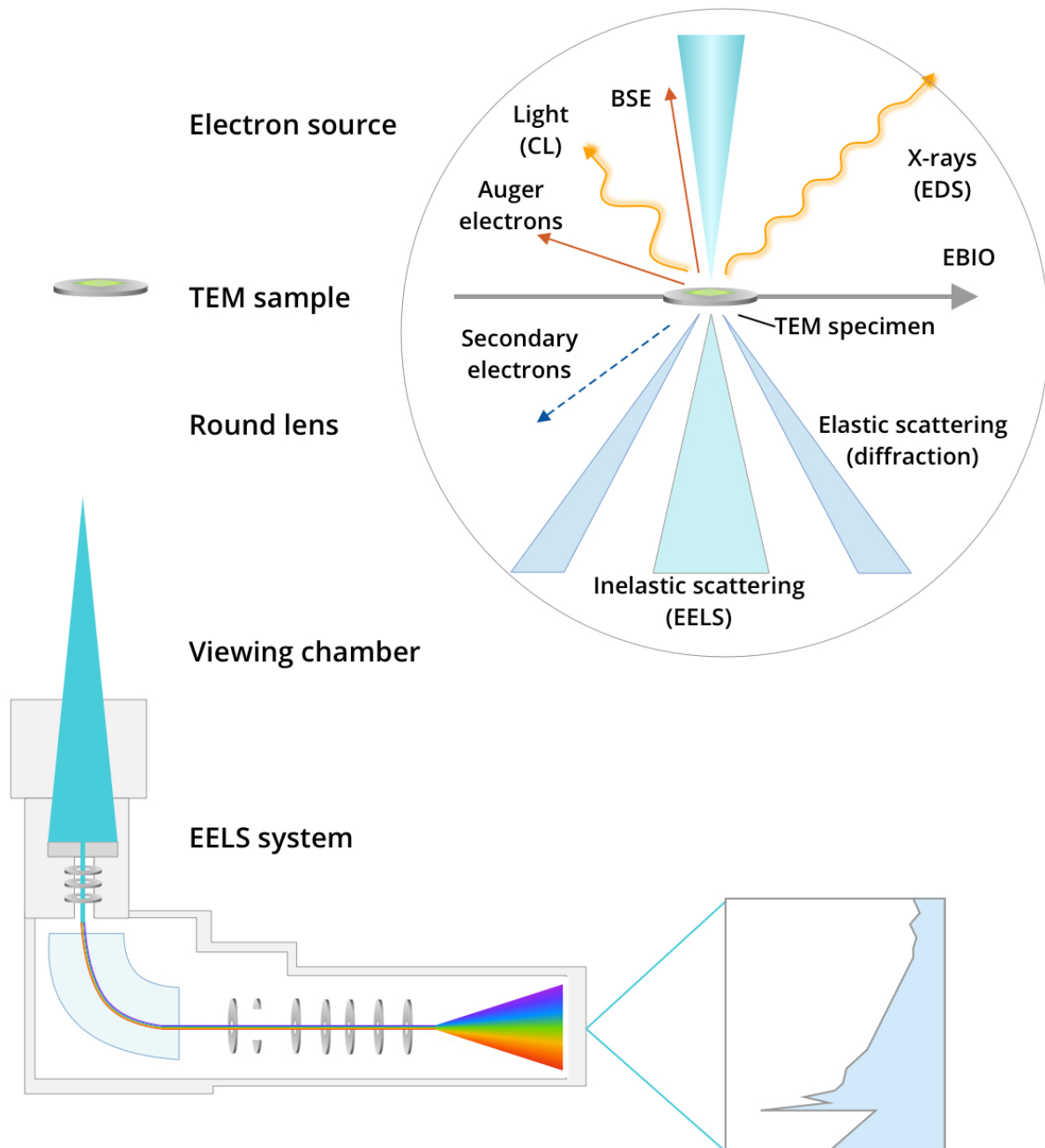
Typically EDS manufacturers supply theoretical k_{AB} values, however proper quantification of EDS data should be performed using standards to determine the correct k_{AB} for each element used.



Comparison of interaction volumes in SEM and TEM

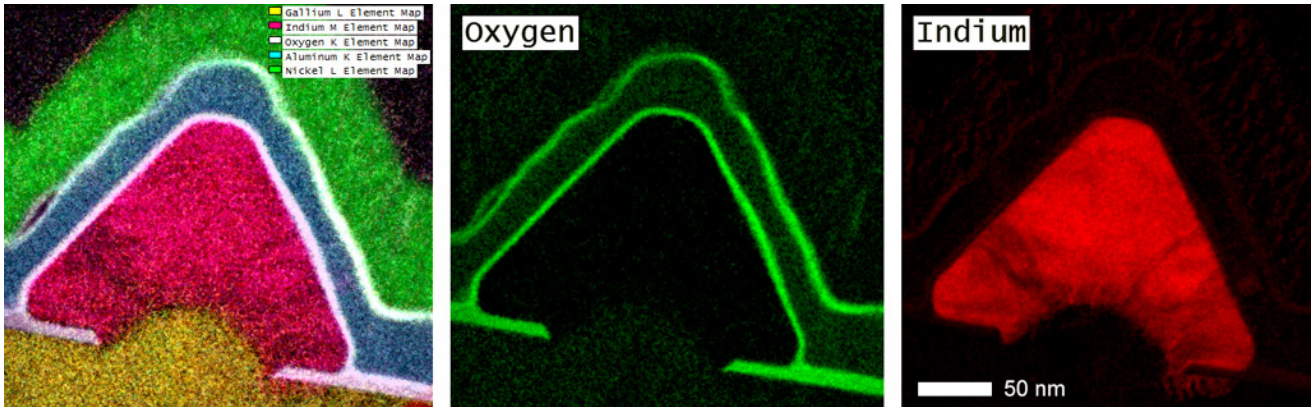
Electron energy loss spectroscopy (EELS)

EELS measures the energy lost by the electrons when they excite the atoms in the specimen to emit X-rays (and other signals). The EELS spectrum therefore contains compositional information that is complementary to that of the EDS signal. EELS can be used for qualitative and semi-quantitative analysis, and has the advantage over EDS that it is very sensitive to light elements down to lithium. The EELS spectrum contains a wealth of other information. For some elements the shape of the spectrum is strongly dependent on the type of bonding in the material. It is therefore possible to determine not only which elements are present, but what type of bonding configuration they are in. Some examples include differentiating between the various forms of carbon or determining the oxidation state of transition series metal oxides.



EDS/EELS spectra can be acquired quickly – requiring just a few seconds to make a qualitative measurement of a single point. Semi-quantitative measurements may take a few minutes. For accurate analysis the specimen must be thin (much less than 100 nm), but this is a general requirement for TEM work.

The EELS signal can also be used to map the chemical distribution of elements over a defined area in a sample. This typically only takes a few minutes compared with perhaps hours for the EDS mapping. EELS-based methods are difficult and considerable expertise is required to apply them effectively.



EELS imaging of a nanowire construction. Images courtesy of Jiangtao Qu, University of Sydney

EDS/EELS are very complimentary, as is scanning TEM (STEM). In STEM, the TEM is operated with a finely focused probe rastered across the specimen. The resulting scanning imaging provides information analogous to that from TEM imaging. However, integration of the STEM system into the spectroscopy software permits the beam to be stopped at any point on the image and an analysis taken. A line profile may be obtained by scanning the beam across of region of interest, such as an interface. A chemical map may be acquired at the same time as the image. This enables distribution of elements within the image to be correlated with microstructural features.

Credits

Microscopy Australia acknowledges the huge input of time and expertise by the many staff members and associates who have contributed to the development of MyScope over the years.

For TEM we thank: Rick Webb, Graeme Auchterlonie, Kathryn Green, Rhiannon Kuchel, Hongwei Liu, Takanori Sato, Naveena Gokoolparsadh, Felipe Kremer, Lyn Waterhouse, Gwen Mayo, Martin Saunders, Alexandra Suvorova, Jeremy Shaw, Ashley Slattery, Nikki Stanford, Jenny Whiting, Garry Morgan.