Chapter 4
Mechanical Preparation Techniques

1 Crushing

1.1 Principle

Crushing is used to very quickly produce a fine powder from a bulk material or fine particle using mechanical crushing.

This technique is essentially used to investigate ceramics, minerals, and mixed–composite materials. Materials must be brittle or made brittle and of a high hardness to average hardness.

1.2 Operating Mode

1.2.1 Equipment and Supplies

The equipment used most often is an agate mortar and pestle. However, earth sciences laboratories usually have automated mortar grinders because the crushing technique is indispensable and is practiced on large quantities of materials. Ball mills are used in other cases; their size and type are adapted to the types of materials to be crushed.

1.2.2 Procedure

The initial material can be in the form of a rough powder obtained by crushing the bulk material or in the form of fibers, platelets (small plates), or single crystals.

Generally, an agate mortar and pestle is used for crushing (Fig. 4.1). A small quantity of the initial material is placed in the mortar, and then it is crushed by rubbing and pressing the material against the mortar with the pestle. This is manual crushing and it is performed on dry material. In some cases, crushing is carried out on moist materials or those containing a solvent such as water or alcohol. This is the case when the initial material is already dispersed in water or alcohol and one does not want to lose its properties by drying it, e.g., in the case of clays derived from granulometric separation techniques (Fig. 4.11). Crushing takes between a few

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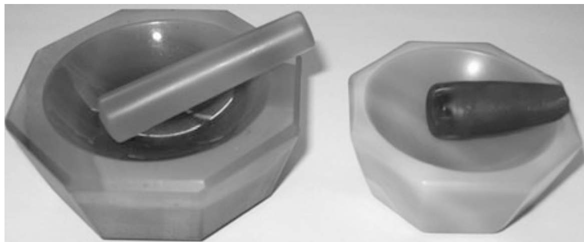

Fig. 4.1 Agate mortars of different sizes. The *darker pestle* is made of corundum (*S. Joulie, IPCMS, Strasbourg*)

seconds and a few minutes depending on material hardness. The powder obtained is placed in suspension in an adequate solvent, and then it is dispersed using ultrasound and spread out using a micropipette onto a support grid containing an appropriate film (either continuous film or holey film depending on the analysis to be performed). The fine powder obtained provides a good representation of the material at the microscopic scale (Fig. 4.11).

In order to prevent contamination of one sample by another, the mortar and pestle must be cleaned after each use. To do this, a scouring powder is placed in the mortar with a bit of water to make a paste, and then the pestle is used to rub the mortar while applying strong pressure. A scrubbing pad with scouring paste can also be used. It is then rinsed abundantly with tap water and given a final rinse with distilled water or alcohol before drying it in a sterilizer. It is recommended to perform a thorough cleaning once in a while by placing the mortar and pestle in a container filled with water into an ultrasound cleaner for an hour or two. It is important to have several mortars dedicated to each type of material, e.g., one mortar dedicated to metallic catalysts, another to ceramics.

In material analysis laboratories, where many investigations are performed, crushing equipment can be more complex or more complete.

To replace manual crushing, there are automatic crushing devices such as the mortar crusher, whose operating principle is the same as an agate mortar and pestle, except that crushing is mechanized.

The device is composed of a rotating mortar, a cover with a hinged opening bearing a freely moving cylindrical pestle, and an adjustable pressure scraper. The sample, moved by the mortar, is detached by the rake, blended, and directed toward the pestle. Crushing is carried out by pressure and friction between the mortar and the pestle (Fig. 4.2). The type of mortar (porcelain, corundum, agate, chromium steel, or stainless steel) and pestle to be used is chosen depending on the hardness of the material to be crushed. Crushing can be carried out dry or with a solvent. These devices are used for larger quantities of material than in the case of a manual agate mortar and pestle.

Crushing quality is more reproducible with automatic crushing than when materials are crushed by hand; a homogeneous powder is obtained down to a micron.

Another type of device widely used in laboratories is the ball mill. The initial material is placed in a sealed cylindrical bowl with ball bearings, and this device can be used for crushing with water or a solvent. The whole device is agitated

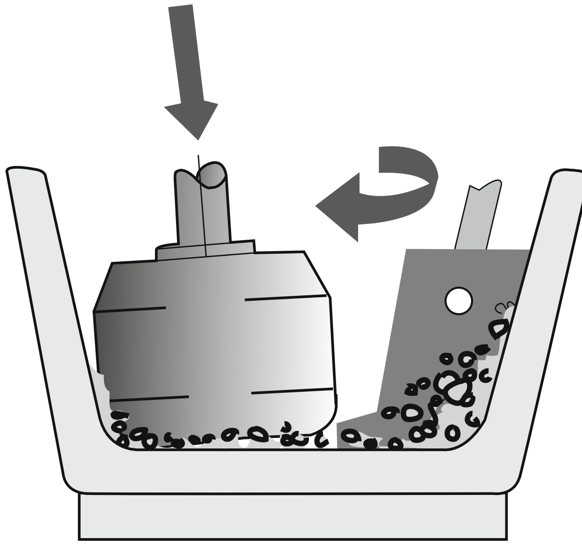

Fig. 4.2 Rotating mortar crusher (*Fisher Bioblock Scientific*)

by an oscillating mixer mill. Crushing occurs by the impact of the bearings that move in three dimensions around the bowl (Fig. 4.3). The size and nature of bearings is adapted to the type of material to be crushed. The bearings are generally made of glass or stainless steel. There are single-use sterile tubes adapted for crushing biological materials. Ball mills are well adapted to crushing small quantities of materials.

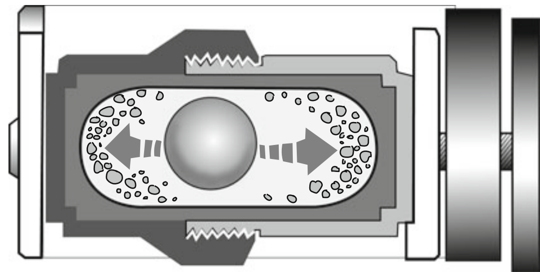

Fig. 4.3 Ball-mill impact crusher (*Fisher Bioblock Scientific*)

1.3 Variants

There are several variants to the crushing technique.

1.3.1 Glass-Slide Technique

The material is crushed by rubbing it vigorously between two clean glass (or quartz) slides, and then it is collected dry on the microscope grid by adherence. This

technique is used when there is a very small quantity of the initial material or when there is a risk of interaction between the material and water or the powder dispersion solvent (Figs. 4.4, 4.5, and 4.12).

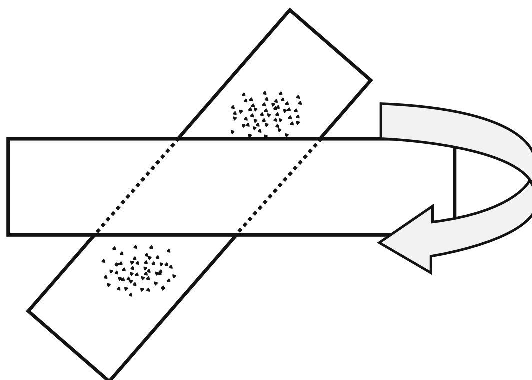

Fig. 4.4 Principle of crushing between two glass slides

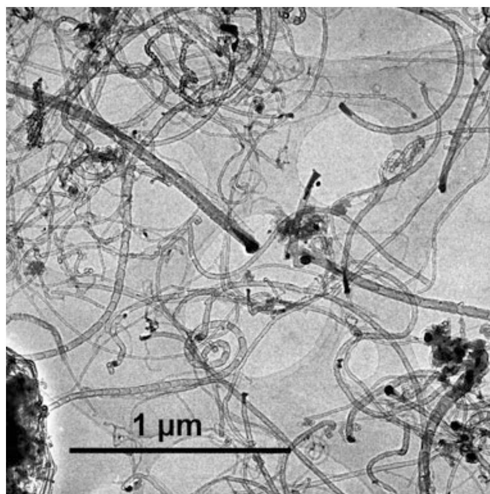

Fig. 4.5 Carbon nanotubes obtained by crushing between two glass slides and recovered dry on a holey film (*C.S. Cojocaru, IPCMS, Strasbourg*)

1.3.2 Cryo-crushing

When the material is not hard enough, it is cooled by immersion in liquid nitrogen to make it hard and brittle. It is then ground in the agate mortar, as in the general case, still in the presence of liquid nitrogen. The powder obtained is deposited onto the support grid, and after the liquid nitrogen evaporates, observation can be made very quickly in the microscope. This technique is used for polymers that are too soft to be crushed at room temperature.

1.3.3 Scraping Technique

This consists of scraping the surface of a substrate supporting a very brittle material. This material is in the form of nanoparticles that adhere strongly to the substrate and are not visible to the naked eye. Using a diamond tip, the surface is scratched in the shape of a small square or pound sign (#). This has the effect of detaching a small film of the substrate with the nanomaterials. It all is recovered by rubbing delicately on the support grid, film side down, at the location of the scratch.

Nanoparticles will adhere to the film, which can be a continuous carbon film or a holey film. This technique makes it possible to preserve the nanostructures in their initial orientation. For example, it is often used for observing carbon nanomaterials obtained by chemical vapor deposition (CVD) growth on substrates such as single-crystal silicon (Figs. 4.6 and 4.7).

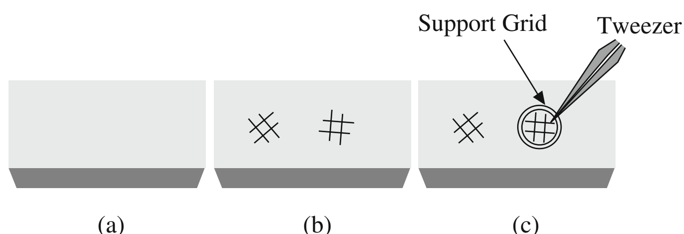

Fig. 4.6 Scraping technique: (**a**) surface to be investigated; (**b**) scratch in the form of a *square* or *pound sign*; and (**c**) sampling on the support grid by lightly rubbing

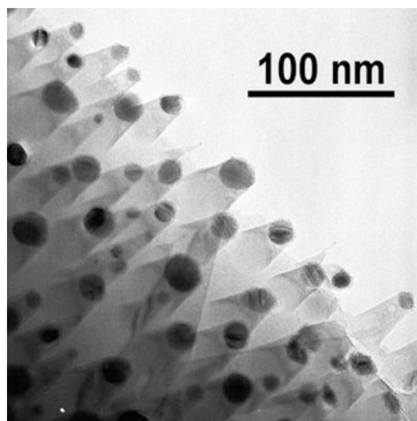

Fig. 4.7 Carbon oxynitride nanopoints obtained by CVD on a single crystal of silicon. Variant of the scraping technique: the material is gathered on a holey film and the orientation of the nanopoints is preserved (*C.S. Cojocaru, IPCMS, Strasbourg*)

1.4 Advantages

Crushing is a simple, fast, and easy technique to use. It is inexpensive, because it does not involve complicated equipment. This technique is widespread in

laboratories, because it is within everyone's grasp and provides a good representation of the initial material. Fine particles presenting several crystallographic orientations can easily be obtained. Crushing does not induce chemical diffusion.

1.5 Limitations

This technique will cause the loss of microstructural organization on a large scale, as the initial material is reduced to a powder. Therefore, it is not used for investigations of isolated interfaces in bulk materials and particularly in materials such as multilayer materials where we are interested in the structure of the interfaces. With this technique, particles with random orientations are obtained (Figs. 4.8, 4.9, and 4.11). Crushing is hard to apply to very hard or very soft materials (Fig. 4.10).

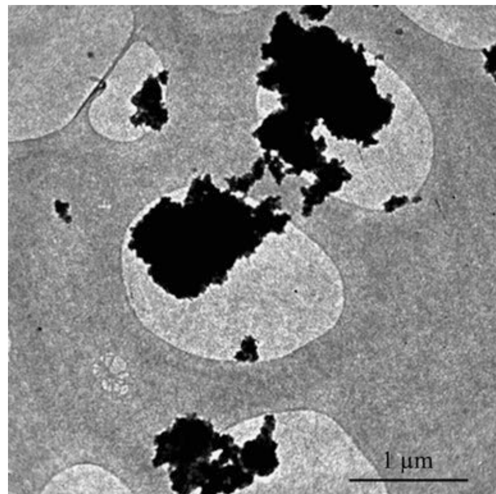

Fig. 4.8 Bright-field TEM image of a catalyst obtained by crushing in an agate mortar, dispersed with ultrasound in distilled water and deposited on a holey film. The material is clumped on the edges of the holes (*G. Ehret, IPCMS, Strasbourg*)

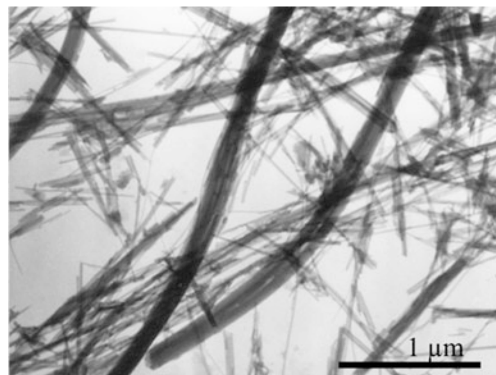

Fig. 4.9 Asbestos fibers (chrysotile) obtained by crushing in an agate mortar, dispersed with ultrasound in distilled water, and deposited on a continuous carbon film. Random fiber orientation (*G. Ehret, IPCMS, Strasbourg*)

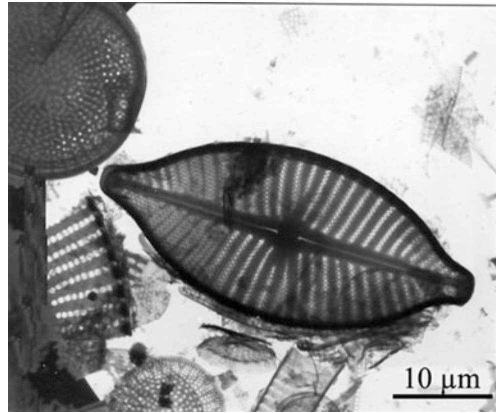

Fig. 4.10 Fossilized diatoms obtained by dry crushing in an agate mortar. Many specimens are broken using this technique (*G. Ehret, IPCMS, Strasbourg*)

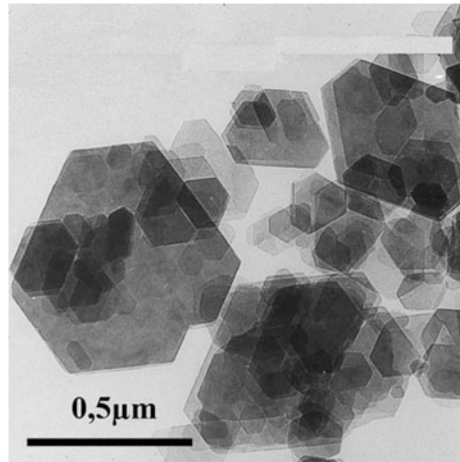

Fig. 4.11 Kaolinite clay particles obtained by crushing in an agate mortar under water and deposited on a continuous film. The platelets (small plates) have a hexagonal facets (*H. Paquet, EOST, Strasbourg*)

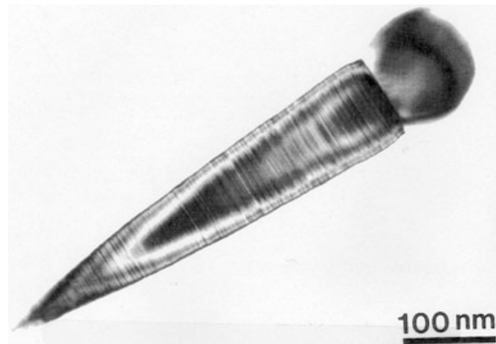

Fig. 4.12 Silicon carbide (SiC) particles in the shape of whiskers. Glass slide variant (*M. Benaïssa, IPCMS, Strasbourg*)

1.6 Artifacts

Crushed materials can present many artifacts: slip planes and dislocations can be introduced, and the initial structure can be lost.

1.7 Type of Analysis

Structure, crystal defects, crystallography, chemical composition, chemical bonds, properties.

1.8 Risks

This technique presents few risks: They are essentially risks tied to inhaling fine dust or noxious vapors from the use of toxic solvents.

1.9 Conclusion

The technique is very widespread in laboratories because it is very easy to use, fast, and inexpensive. It is often the first approach for investigating a wide range of materials and can be sufficient in many cases such as investigating minerals or catalysts. It is the technique of choice for fine particles. For bulk materials, the other possible techniques are ultramicrotomy, tripod polishing, or ionic thinning.

2 Wedge Cleavage

2.1 Principle

The wedge-cleavage technique is used to quickly obtain a thin slice, generally of a multilayer material, using a cross-sectional cut. This involves obtaining a sample of approximately 0.6 mm^2, presenting a perfect edge (without damage). The edge is obtained through a succession of fractures by cleavage along an atomic plane of a material sample whose substrate (as well as the layers for an epitaxial multilayer) is monocrystalline and, of course, cleavable. The far side of the edge is then transparent to electrons over a distance that extends from the surface of the sample up to the base of the substrate.

The sample is then mounted on a support that allows proper orientation of the sample relative to the electron beam (Figs. 4.13 and 4.14).

Cleavage is facilitated by the presence of planes that are weakly bonded to one another; however, it depends on the quantity of atoms in a plane and on the type of bonds between the atoms. Figure 4.15 shows the normal cleavage planes of a gallium

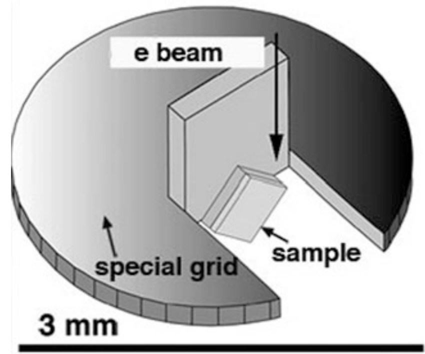

Fig. 4.13 Mounting diagram for the cleaved wedge on the special support disk that makes it possible to orientate the wedge in the TEM

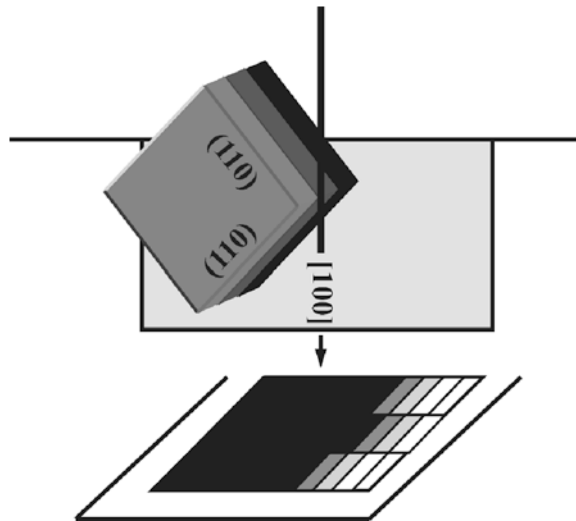

Fig. 4.14 Orientation of the cleaved GaAs wedge for observation in the TEM and the projected image of the wedge

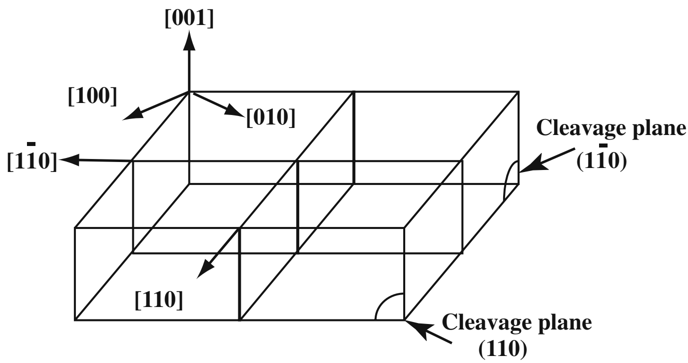

Fig. 4.15 Example of cleavage planes for a GaAs single crystal

arsenide (GaAs) substrate. Cleavage is facilitated by the generation of dislocations created by micro-cracking at the initiation of cleavage (Fig. 4.16).

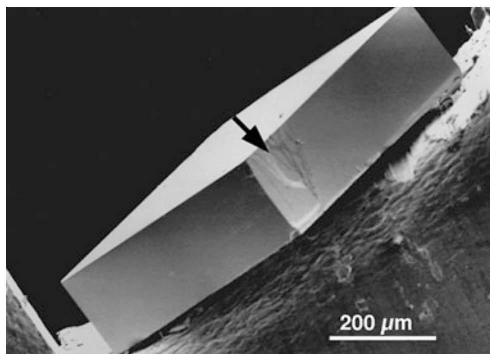

Fig. 4.16 The *black arrow* shows the micro-cracks generated by the initiation of cleavage. Scratching the sample surface with a diamond scriber does cleavage initiation. Secondary-electron SEM image

This technique is applied to multilayer materials deposited on a substrate, whether they are crystalline, polycrystalline, or amorphous.

2.2 *Operating Mode*

2.2.1 Equipment and Supplies

The only equipment necessary for the technique is a hot plate heated to approximately 373 K in order to quickly polymerize the epoxy adhesive. The tools and supplies needed are listed below:

- One piece 600-grade (18 μm) SiC sandpaper and one piece 2,400-grade (10 μm) SiC sandpaper
- Soluble thermal glue
- Cotton swabs for cleaning the sample, if needed
- Pure acetone
- Pure ethanol
- Two very fine diamond scribers, one of average quality for reducing large samples, the other of top quality for cleaving wedges
- One wide-ruled block (approximately 5 mm) of graph paper
- One small, thin, flexible plastic ruler
- One steel cylinder approximately 2 mm in diameter, approximately 10 mm in length
- Round filter paper approximately 5-mm thick
- One piece of transparent plastic, approximately 40 mm × 40 mm (e.g., plastic used for covering notebooks)
- One vacuum tweezers for handling small samples
- Four self-closing tweezers

– One fine-point tweezers
– Four special supports (aluminum, copper, etc.) for wedges or other mounting grids
– Epoxy (e.g., Araldit Rapid)

2.2.2 Procedure

Cleaving a Strip from a Wafer

A wafer always has one cleavage direction indicated by a plane edge (Fig. 4.17), but most times, the laboratory receives only a part of the wafer already cleaved.

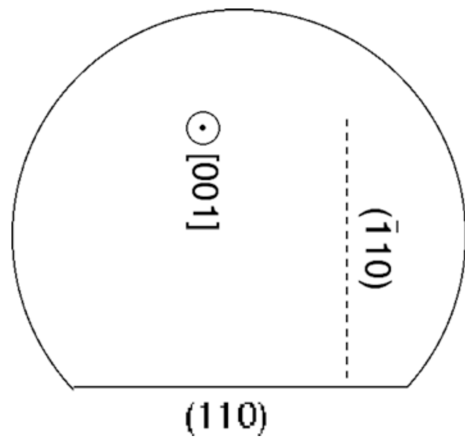

Fig. 4.17 Initial wafer with the indication of the cleavage plane

In order to begin the technique, a sample piece of the wafer approximately 0.5 mm × 10 mm is recommended.

Reducing the Sample Size Through Successive Cleaving

The sample is laid on graph paper, with the surface to be analyzed (layers) facing upward (Fig. 4.18). Using the diamond tip, a micro-crack must be scored approximately 2 mm long, at 90° from the plane face, using a small flexible plastic ruler to guide the diamond scriber (Fig. 4.19).

Note: For silicon, the micro-crack must be longer and deeper than for other materials.

Scoring with the diamond scriber is done on a good surface, generally flat, ensuring a thin straight line (Fig. 4.20).

Note: The pressure applied to the diamond scriber should be light, so as not to score too deeply and too widely, which could result in cleavage in a random direction.

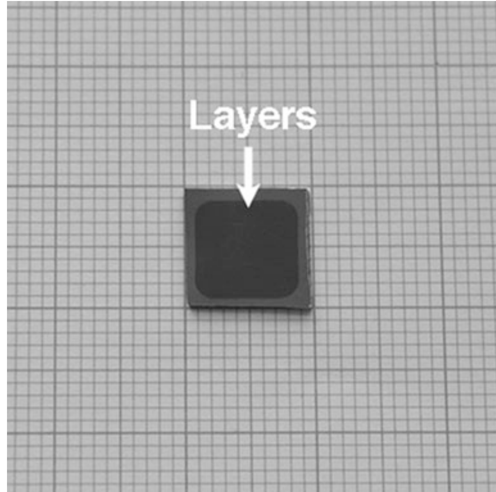

Fig. 4.18 Layers are facing upward

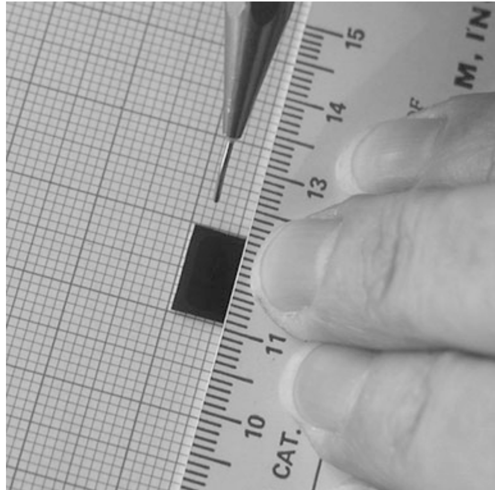

Fig. 4.19 Scoring micro-cracks using a diamond scriber

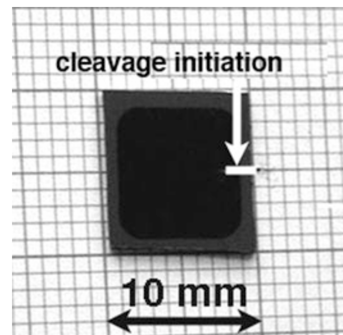

Fig. 4.20 Score mark is 1- to 2-mm long

The sample is then returned to the graph paper, covered with a plastic sheet, and pressure is exerted on it by lightly rolling the metal cylinder in the area of the micro-crack until the sample cleaves (Fig. 4.21).

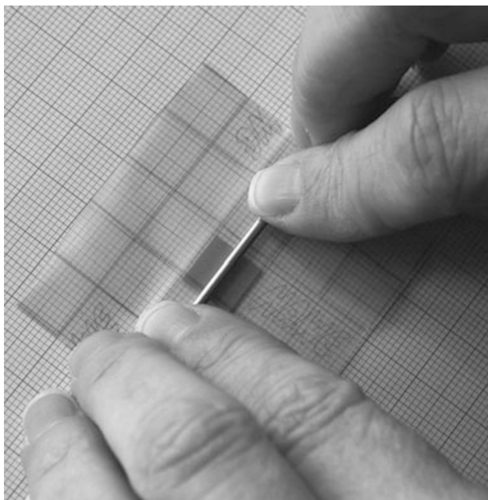

Fig. 4.21 Cleaving the sample using the metal cylinder

If necessary, other cleavages must be performed, so as to obtain a strip of approximately 5 mm × 10 mm (Fig. 4.22).

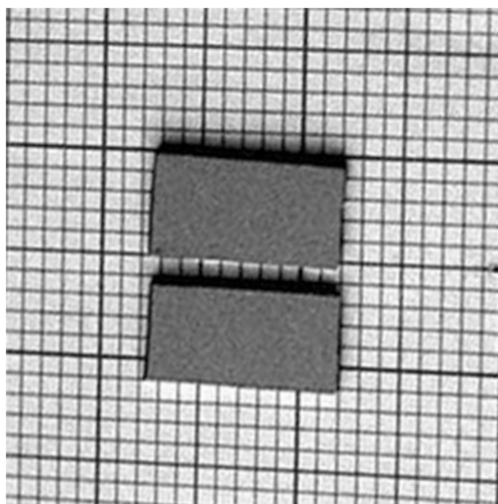

Fig. 4.22 Result after the first cleavage

Reducing Substrate Thickness Through Mechanical Polishing

The strip thickness is reduced by mechanical polishing in order to facilitate cleavage. Residual thickness depends on the material. For example, a GaAs sample whose growth direction is [100] easily cleaves if the thickness is 150 μm, whereas a Si sample whose growth direction is [111] must be reduced to a thickness of around 80 μm.

A sample strip is glued to a support for polishing, with the face to be observed against a thermal glue.

Mechanical abrasion is carried out on coarse SiC sandpaper (600 grade) until the desired thickness is reached. A finer polishing using 2,400-grade SiC sandpaper is then carried out. It is important not to perform too fine a polishing in order to be able to then distinguish the abraded surface from the surface containing the layers.

The sample is unglued from the support so it can be washed in successive baths of acetone, followed by ethanol and isopropanol.

Note: If traces of solvent are left behind on the surface containing the layers, it is possible to eliminate them by brushing them delicately with a cotton swab soaked in pure acetone, then ethanol. *Do not use this procedure if there is a risk of damaging the surface (e.g., on a metal-plated surface).*

Cleaving

The strip is once again lined up on the graph paper, with the layer side on top, and two scribe lines 1-mm long are then made no less than between 0.5 and 0.7 mm apart (Fig. 4.23). *Comment*: During mechanical polishing, the edge of the strip is damaged and is not usable.

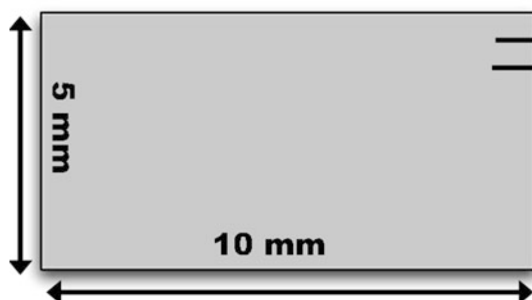

Fig. 4.23 Preparing a 0.5- to 0.7-mm wide strip

Note: The pressure applied to the diamond tip should be light so as not to score too deeply and too widely, which could result in cleavage in a random direction.

The strip is turned over and then laid on a layer of filter papers or any other material that will give it lightly cushioned support. A sheet of plastic is laid over the sample (to prevent it from jumping up) and the cylinder is rolled from bottom to top around the diamond scores, while applying light pressure.

The resulting strip is again placed on the graph paper and several small score marks are made at a distance between 0.5 and 0.7 mm apart (Fig. 4.24), in order to produce several samples. To make two observable wedges on the same sample, the score mark must be made once on the left and once on the right as shown in Fig. 4.24. As a precaution, it is recommended that three to four wedges are made from the same sample.

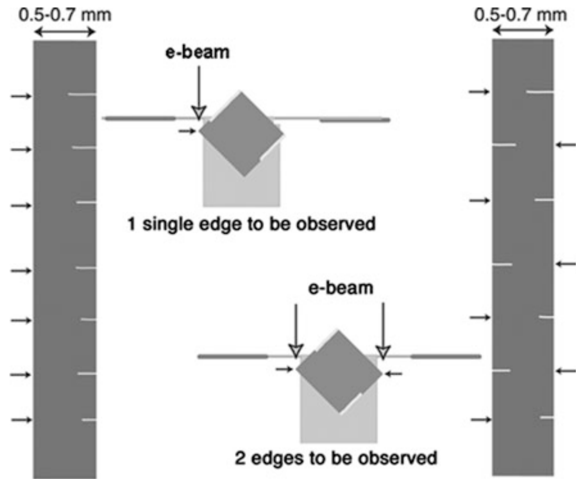

Fig. 4.24 To get two observable wedges on the same sample, the score mark must be made once on the left and once on the right

The strip is turned again and cleaved as before.

Wedge Selection

Small samples must then be turned over and laid on a glass slide to be observed in an optical microscope (Fig. 4.25). They are transported onto the glass slide using a pair of vacuum tweezers. The use of tweezers, as fine as they may be, runs the risk of damaging the sample corners that must have a perfect edge.

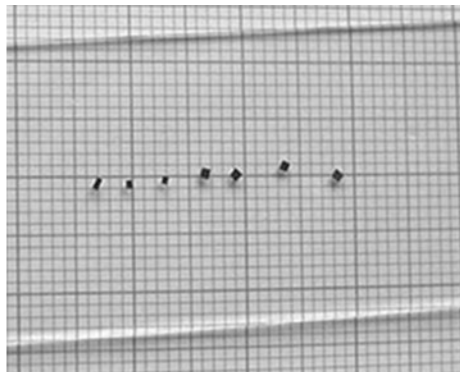

Fig. 4.25 Cleaved wedges are placed on a glass slide, layer-side up, in order to enable selection of the perfect wedge

An optical microscope is used to select corners that have a perfect edge. Corners that have been damaged by scoring are clearly visible and will not be considered when selecting a perfect corner. The selected corner should have a perfect 90° angle and should not present any small residual dust either along its edge or on its surface. This dust would hinder or make TEM observation impossible (Fig. 4.26).

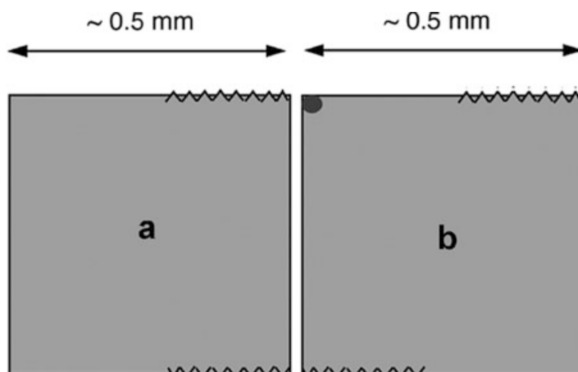

Fig. 4.26 (**a**) Both corners on the *left* are usable; (**b**) the dark spot in the *upper left-hand corner* represents dust deposited on the surface, making the edge unusable. Only the corner at the *bottom right* can be observed

In order to be able to recognize the corner selected when mounting the sample on the support for the TEM, using a stereoscopic microscope, it is recommended to mark this corner using a felt-tip pen on the glass slide near the selected corner.

Gluing the Sample to the Support Disk

Several types of support can be used to mount the sample in the configuration necessary for TEM observation. A full 3-mm disk can be cut into the sample in two places, 1-mm apart, from the edge to the middle; this part can then be bent to a 90° angle. A more elaborate support can be produced by electrical discharge machining (EDM) (Fig. 4.27).

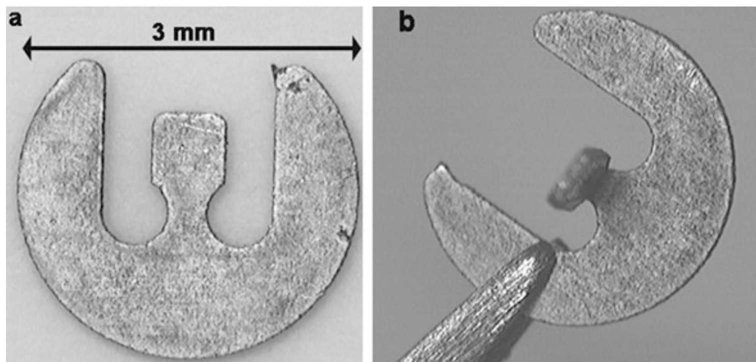

Fig. 4.27 (**a**) A special support cut using electromachining and (**b**) after bending the tab to a 90° angle

If chemical analysis of the sample is necessary, a support made from a material that is not found in the sample's chemical composition can be used.

The cleanliness of the disk and the flatness of the tab must be checked under a stereoscopic microscope.

The disk is held using self-closing tweezers and the tab is laid horizontally. (*Comment*: Use a pair of strong self-closing tweezers to hold the support securely.)

A thin layer of epoxy (Araldit Rapid) is deposited onto the support tab (Fig. 4.28) under the stereoscopic microscope. It is essential not to put down too much glue in order to prevent excess glue from moving by capillary action along the edge of the corner, making it impossible to observe the sample in the TEM.

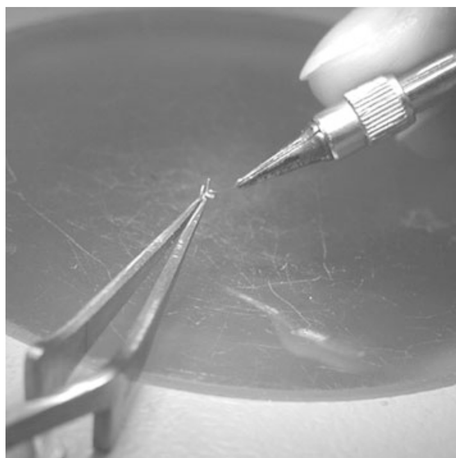

Fig. 4.28 Depositing a small drop of glue

The sample is placed on the tab using a vacuum tweezer (Fig. 4.29) in the following way (Fig. 4.30):

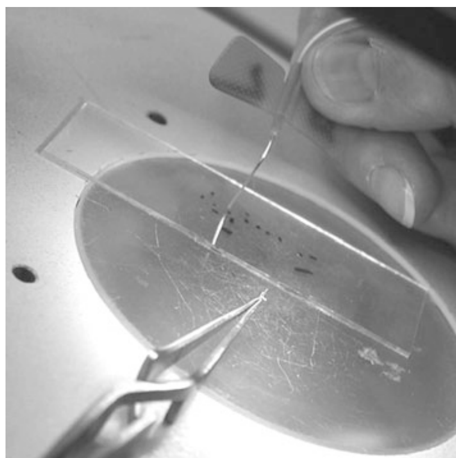

Fig. 4.29 Sample is moved and glued onto the special support

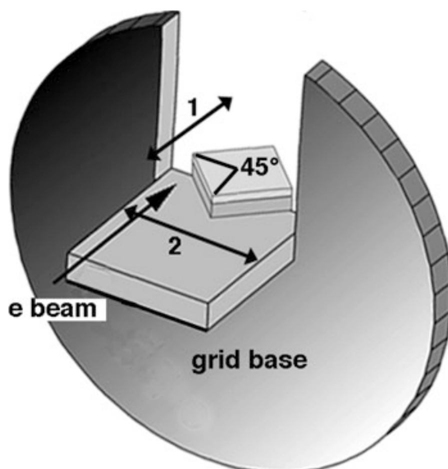

Fig. 4.30 Mounting the wedge onto the support disk. The angle between the incident beam and the edge of the specimen should be 45°

– The wedge(s) to be observed are placed as close as possible to the base of the disk in order to be close to the eucentric height in the microscope (#1 in Fig. 4.30).
– The wedge must be in the middle of the tab in order to prevent shadowing of the beam (#2 in Fig. 4.30).
– Position the wedge at a 45° angle to the incident electron beam. If the wedge is positioned too far off this angle, sample observation cannot be parallel to the layers (on the axis of the area), given the limited tilting angles of the microscopes (in general) as well as the risk of masking the incident beam by the support grid.

In order to reduce the polymerization time of the glue, the tweezer-grid-sample setup can be placed on a hot plate for 10 min at a temperature of approximately 373 K.

Mounting the Preparation in the Microscope Specimen Holder

Once the sample is mounted on the TEM specimen holder, it must not go above or beyond the thickness of the TEM specimen holder in order to prevent any risk of damage to the pole piece of the microscope, which is situated just above the sample housing.

2.3 Variants

2.3.1 Adapting the Method for the Small Angle Cleavage Technique (SACT)

For some materials (silicon, sapphire, glass, etc.), a low-angle cleaved wedge can be prepared in order to produce a thin area perpendicular to the edge larger than would

be made with a 90° cleaved wedge. In this case, it is no longer a matter of separating the sample based on the weakest bound planes, but imposing another cleaving direction in the desired plane. This type of preparation is made by "removing" a determined quantity of material by rough polishing, on the back of the substrate, in a unilateral direction corresponding to the direction of the planes to be cleaved. This polishing is supplemented by removing material with a diamond tip along the already premade grooves, and then by cleaving. Figures 4.31, 4.32, 4.33, and 4.34 show a few preparation steps of a silicon sample at an angle of 18.43°, i.e., between the (110) and (120) planes.

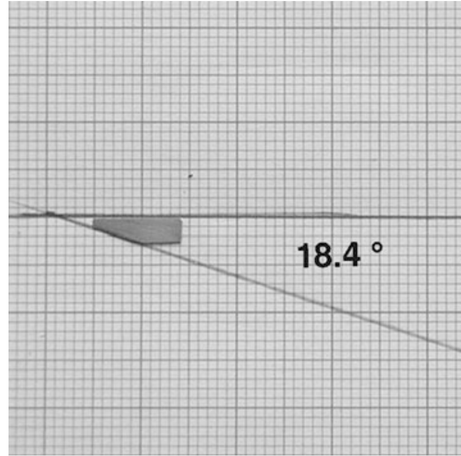

Fig. 4.31 First cleaving at 18.4° of the initial cleavage plane after unilateral mechanical polishing in the cleavage direction

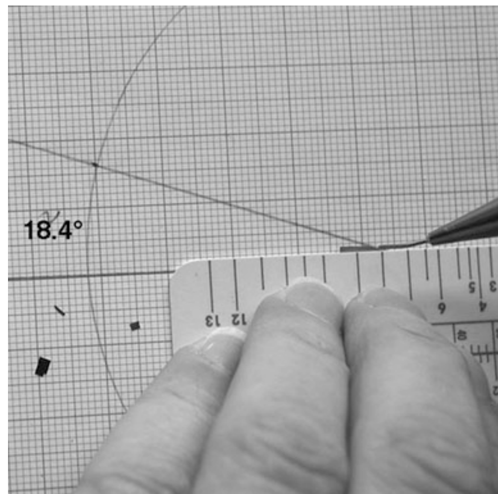

Fig. 4.32 Second cleaving along the initial cleavage plane

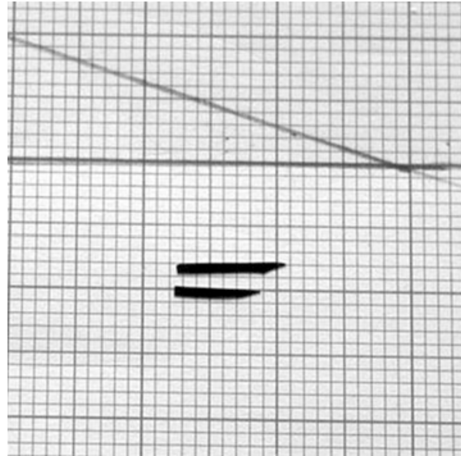

Fig. 4.33 Result after two successive cleavings

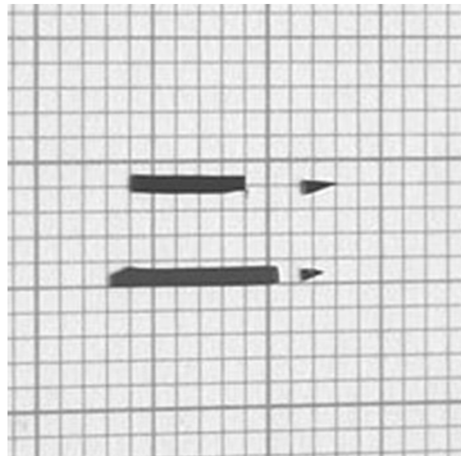

Fig. 4.34 Result after cleaving perpendicular to the initial cleavage plane for producing a small sample

The resulting wedges are then mounted on a support adapted for observation.

The small-angle wedge cleavage method is thoroughly detailed by Scott D. Walck and John P. McCaffrey (*Mat. Res. Soc. Symp. Proc.,* **480** (1997))

2.4 Advantages

The technique does not induce any chemical diffusion and allows for qualitative chemical analysis. *This is the only technique that enables semi-quantitative chemical analysis* from the investigation of equal-thickness fringes, visible along the edge of the wedge. In fact, for epitaxial layers, the thickness (at the atomic scale) of the

cleaved wedge is constant all along the edge. The interfaces are revealed by the discontinuity of the equal-thickness fringes, whose profile depends directly on the chemical composition.

Contrast analysis of equal-thickness fringes, combined with a simulation of a theoretical model, can be used to determine the relative chemical composition of the layers (Fig. 4.35).

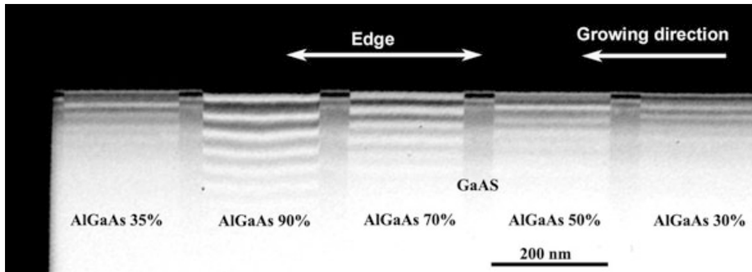

Fig. 4.35 Bright-field TEM image of an AlGaAs/GaAs multilayer. A TEM image combined with a simulation program is used to make a semi-quantitative analysis of the chemical composition from the investigation of equal-thickness fringe contrast. The percentages indicated on the TEM image correspond to the amount of aluminum calculated from this image analysis (*D. Laub, EPFL-CIME, Lausanne*)

The method is also used for materials whose layers are not epitaxial but are deposited on a cleavable substrate, e.g., a Cr/Ti multilayer deposited on a Si substrate (Fig. 4.36) or a Au/SiO_2 layer on a Si substrate (Fig. 4.37). In this case, the layers are not cleaved but fractured (more or less) along the cleaved edge of the substrate. Layer thickness is not constant along the edge, but the cleaved wedge

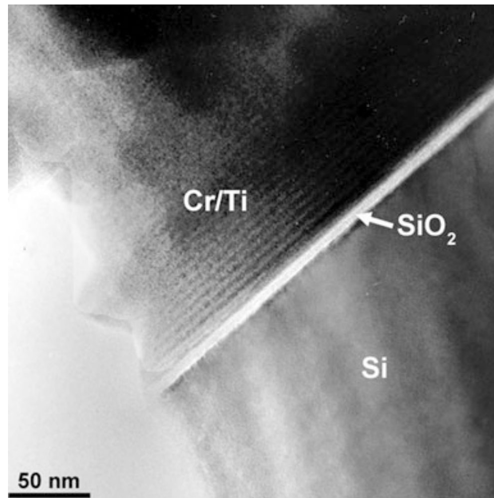

Fig. 4.36 Layers of Cr/Ti-SiO_2 on silicon [111]. The cleaved wedge is used to quickly show the multilayer growth of the Cr/Ti layers near the substrate (*D. Laub, EPFL-CIME, Lausanne*)

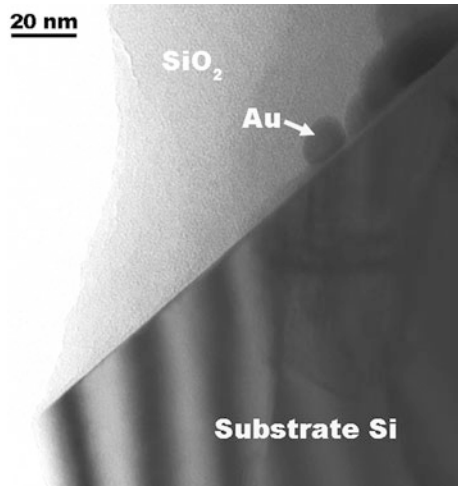

Fig. 4.37 Bright-field TEM image. A SiO_2/Au particle layer on silicon [111] (*S. de Chambrier, A. Schüler, EPFL-LESO-PB, Lausanne*)

makes it possible, for example, to measure layer thickness of a multilayer material or to verify the structure of a material in order to make sure it has not undergone any transformation during preparation using another technique such as mechanical polishing or ion milling.

A bulk material can also be prepared, e.g., in order to follow the diffusion profile of an element within the material (Fig. 4.38) or even to investigate the density and implantation depth of ions or particles in a material.

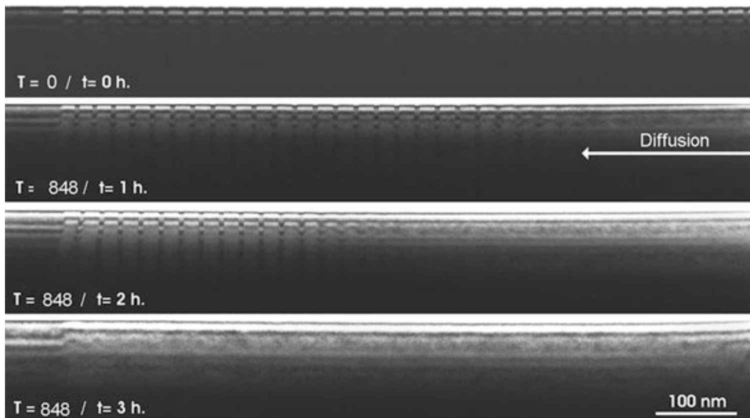

Fig. 4.38 Disorder induced by the diffusion of impurities (Zn) in an AlGaAs semiconductor. Impurity diffusion within a quantum-well structure is accompanied by diffusion of elements from group V (Ga and Al) and consequently can change the chemical composition in a specific area of the structure. The wedge cleavage technique is used to "monitor" the disorder induced based on the temperature and duration of diffusion. Bright-field TEM image (*J.-D. Ganière, EPFL-IPEQ, Lausanne*)

Table 4.1 Examples of cleavage planes for different crystals

Crystal	Cleavage planes	Angle of the cleaved wedge
GaAs	(110)	90°
Si	Primary (111)	70.53°
	Secondary (110)	90°
	(110) and (120)	18.43°
	(111) and (110)	35.26°
MgO	(100)	90°
NaCl	(100)	90°

The wedge cleavage technique is an easy technique to use in terms of cleaving a wedge at an angle of 90°, but is more delicate when preparing a small-angle wedge.

Table 4.1 shows the cleavage planes of a few single crystals.

Approximately 1 h is needed to prepare four wedges from the same material. It is inexpensive; the two diamond tips are the main investment. There are no artifacts due to the preparation, as with mechanical polishing defects or defects caused by ion milling. This technique is used to measure the thickness of all of the layers deposited on a substrate, as well as high-resolution analysis.

A non-exhaustive list of the different materials prepared using the wedge cleavage technique is provided in Table 4.2.

Table 4.2 Examples of materials prepared by the wedge cleavage technique

Cerium fluoride	CeF_3	Lithium tantalate	$LiTaO_3$
Lithium tantalite	$LiTaO_3$	Cobalt silicate/silicon	$CoSi_2/Si$
Vanadium selenide	VSe_2	Zinc oxide-gold/silicon	ZnO-Au/Si
Titanium disulfide	TiS_2	Chrome-cobalt/silicon	Cr-Co/Si
Potassium tantalate	$KTaO_3$	Tungsten/silicon	W/Si
Potassium niobo-tantalate	K $TaNbO_3$	Diamond-like carbon/silicon	DLC/Si
Lead titanate	$PbTiO_3$		

2.5 Limitations

This technique is limited to cleavable materials. The observable area does not exceed 200 nm perpendicular to the edge, limiting interface investigation. The ascending thickness in the direction perpendicular to the edge does not allow for quantitative spectroscopic analysis using EDS or EELS.

2.6 Artifacts

This technique generates few or no preparation artifacts. However, it is limited to cleavable materials, with regard to the substrate. Bad cleaving or a strained sample can produce dislocations or dislocation clusters (Fig. 4.39).

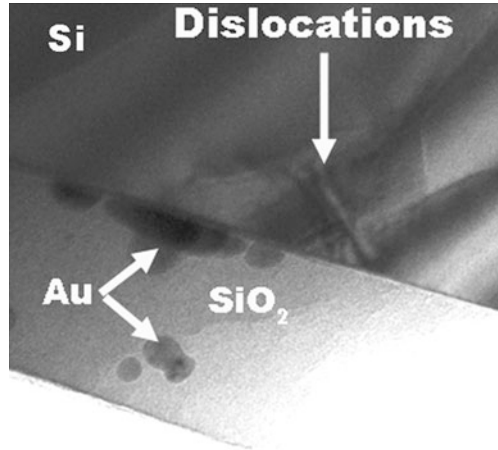

Fig. 4.39 Dislocations in the silicon, probably generated by cleaving (*S. de Chambrier, A. Schüler, EPFL-LESO-PB, Lausanne*)

In the case of stresses in the layers, it becomes difficult (even impossible) to associate a disturbance of equal-thickness fringes with a change in chemical composition

2.7 Type of Analysis

Structure, crystal defects, crystallography, chemical composition.

2.8 Risks

Protection must be used against inhaling fine dust created by scoring when preparing noxious materials such as GaAs.

2.9 Conclusion

Wedge cleavage is the method of choice for quickly checking the growth of epitaxial heterostructures. In this case, it is the only method allowing for investigation of chemical composition using equal-thickness fringes. For interface investigation, it

is preferable to use the ionic thinning technique (Chapter 3, Section 5) or the tripod-polishing technique (Chapter 4, Section 3).

3 Tripod Polishing

3.1 Principle

This technique is used to prepare a beveled thin slice. It involves polishing by rubbing a gentle abrasive on a slightly angled sample (0.3–0.7°), resulting in optical-quality surfaces whose wedged edge is electron transparent (Fig. 4.40).

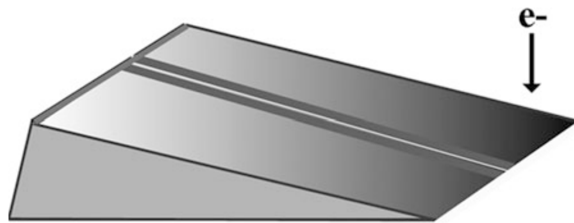

Fig. 4.40 Diagram of a multilayer material "sandwich" prepared for cross-sectional observation. Bevel polishing

When the sample has been reduced to the required dimensions or prepared using the sandwich method, it is mounted onto the tripod polisher to plane polish the first side of the sample.

Polishing is performed on a succession of plastic disks embedded with diamond grains of decreasing grain sizes (30, 15, 6, 3, 1, 0.5 μm, and sometimes 0.1 μm).

Final polishing to eliminate any surface scratches and reduce the thin area when polishing the second side of the sample is done on a soft felt pad soaked with a colloid. This solution contains very fine grains (0.025–0.05 μm) of silica, alumina, diamond, etc.

The sample is then unglued from the support. It must be polished perfectly flat in order to give the sample a proper wedge angle when polishing the second side. The polishing steps for the second side are exactly the same as those for polishing the first side.

Lastly, the sample is mounted onto a support washer with a diameter appropriate for the microscope specimen holder (Fig. 4.41).

The tripod method can be performed on any type of compact or porous, single-phase or multiphase material, with the exception of soft, very soft, or ductile materials. They can be electrically conductive or insulating. Porous materials must be epoxy infiltrated beforehand, and fine particles must be embedded beforehand.

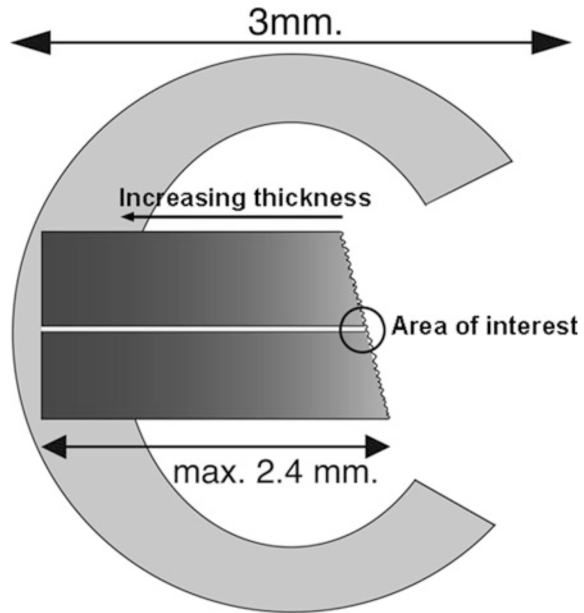

Fig. 4.41 Overhead view of a cross-sectional sample angle polished and mounted on the support washer

3.2 Operating Mode

3.2.1 Equipment and Supplies

This technique contains four distinct elements: a rotating polisher on a table, a sample support held by the operator, an optical microscope for checking polishing quality and measuring the depth of abrasion, and a stereoscopic microscope for gluing the thin slice to a support washer at the end of the process.

The slow-speed rotary polisher is equipped with a glass turntable to which the abrasive disk is attached.

The glass or Pyrex sample support can be tilted using three micrometric screws, which is why it is called a tripod (Fig. 4.42). Abrasion is generally performed under water or in a solvent.

The tripod consists of the following**:**

- One central body
- Three Teflon or Delrin feet adjusted using three micrometric screws with a precision of 10 μm
- One removable L-bracket containing a glass or Pyrex cylinder as a sample support (enabling transmitted-light observation)
- One spirit level
- Three locking screws for locking the micrometer screws

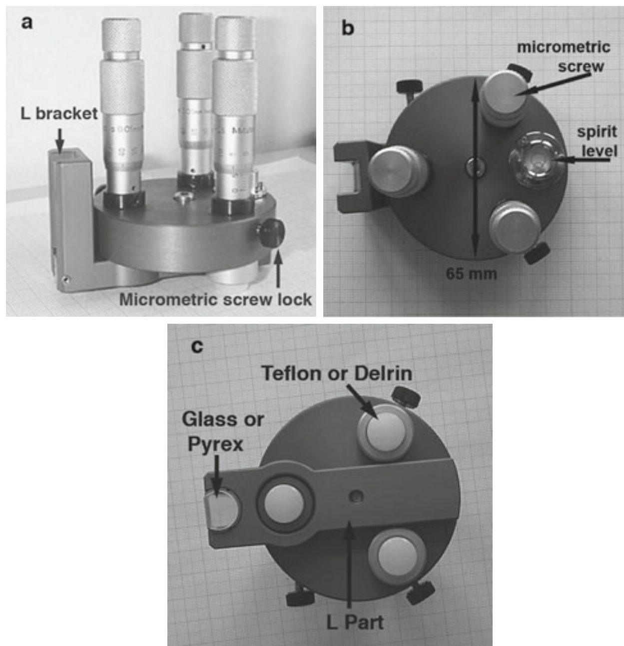

Fig. 4.42 The tripod polisher, seen in (**a**) profile, from (**b**) overhead, and (**c**) underneath

The micrometer screws:

1. keep the sample level and control the depth of abrasion for polishing the first side. All three micrometer screws are used to do this
2. introduce the angle to the sample wedge for polishing the second side and, if necessary, correct the angle during preparation. Only the tripod's two rear micrometer screws are then necessary

A fundamental aspect of this type of polishing is constant observation of the polishing surface during the different steps, in order to check polishing quality and measure sample thickness variation with a precision of a few micrometers.

Two microscope systems can be used: an inverted (metallographic) microscope, upon which the tripod is placed, and a non-inverted microscope with a modified stage for placing the tripod tool (Fig. 4.43). Thickness measurement can be done using either the gradation of the microscope's lined focusing screw or a digital indicator mounted on the microscope. In both cases, it must be possible to measure thickness by unfocusing the objective lens (Fig. 4.44).

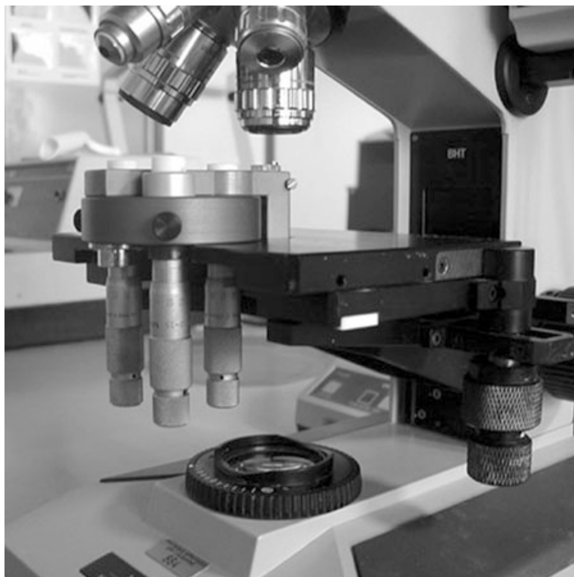

Fig. 4.43 Microscope with modified stage for placing the tripod tool

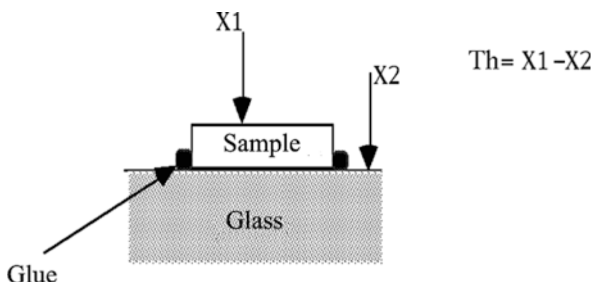

Fig. 4.44 Measuring sample thickness (Th)

3.2.2 Procedure

The procedure described below is used for a cross section. It can be adapted to wedge preparation of a flat section or preparation of a polishing plane cross section (see Section "Adapting the Tripod Method for Plane Polishing (Not Beveled) of a Cross Section").

Cutting the Sample

The sample must be cut to the required dimensions by sawing, cleaving, etc. For a wedge cross-section, prepare two sample strips approximately 10 mm long (if the sample quantity so allows) and 1.5–1.7 mm wide, for a thickness of up to approximately 500 μm per strip. These two strips are stuck together using the sandwich technique.

Mounting the Sample on the Tripod

The tripod's L-bracket is heated along its short section. The sample is stuck using thermal glue to the flat part of the glass cylinder. It must extend beyond the lower end of the glass cylinder by approximately 2 mm in order to allow abrasion and must be as parallel as possible to the edge of the glass (Fig. 4.45). If the starting sample is not longer than 500–800 μm, it is glued to the lower part of the glass support (Fig. 4.46).

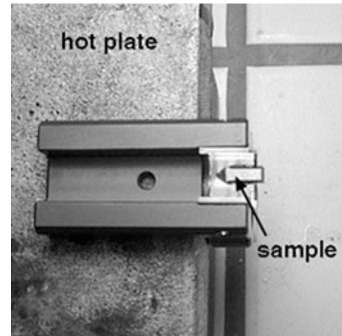

Fig. 4.45 Overhead view

Fig. 4.46 Small sample stuck to the lower part of the glass cylinder

The L-bracket is mounted on the body of the tripod and the tripod is placed on a completely flat, level table. The sample and the two rear feet are in contact with the table (Fig. 4.47). The two rear micrometer screws must be set so that the side of the sample to be polished is parallel to the table (Fig. 4.48), and then the third

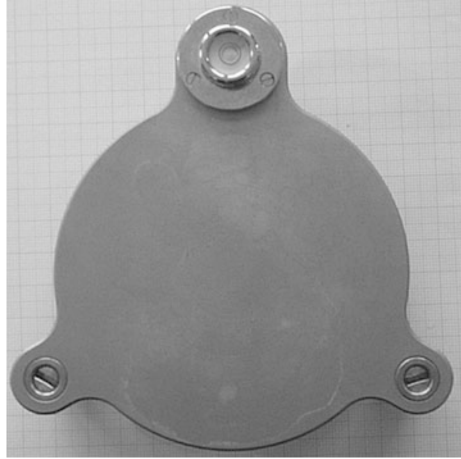

Fig. 4.47 Leveling table

micrometer must be lowered so that it touches the table top. The moment of impact between the screw and the base can be felt, or the air bubble in the level can be seen to move: the sample and the micrometer screws are now in the same plane.

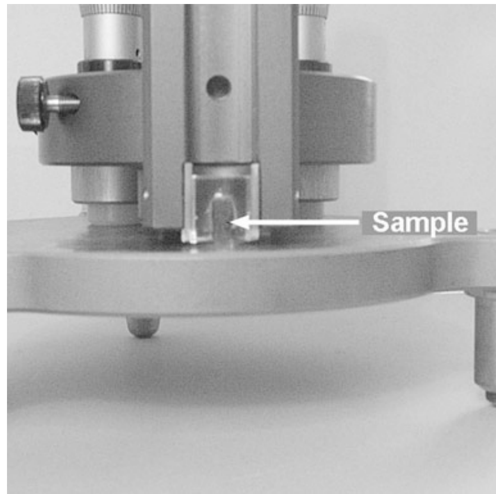

Fig. 4.48 Leveling the rear feet and the sample

To begin polishing, the three micrometer screws must be retracted. The initial cutting method is then accounted for. If the sample was cleaved, the induced damage is not very deep; however, if the sample was sawed it must be polished deep enough to ensure that the defects generated by the grain size of the saw are eliminated. Generally speaking, the depth of damage induced during mechanical damage can be calculated as three times the grain size of the abrasive used. This amount depends on the type of material and is just a recommended guideline value (see Table 4.3).

Table 4.3 Rotational speed and abrasion depth required based on the diamond grain size

Grain size (μm)	Actual rotational speed[a] (rpm)	Minimum abrasion depth (μm)
30	50–75	Dependent on the sample cutting method
15	20–30	90
6	10–12	45
3	Minimum speed	18
1	Minimum speed	9 retract the front micrometer
0.5	*Without* rotation, polish the sample in a straight-line movement	Until good surface quality
Colloidal silica or other, 0.05 or 0.025 μm	100	Until a scratch-free surface is obtained (approx. 1½ min)

[a]Rotational speed values indicated on rotary polishers may differ from actual values.

For example, if sawing was performed with 60-μm abrasive grains, the sample must be abraded with the first polishing disk to a depth of 180 μm. Consequently, the micrometer screws must be retracted by 180 μm.

Preparing the Rotary Polisher

The rotary polisher must be thoroughly clean and abrasive disks must be cleaned. The diamond disk is affixed to the glass disk that has been moistened with tap water using a "squeegee" or a rubber rectangle.

Polishing the First Side

The different polishing steps include a succession of polishings with diamond abrasive disks, requiring varying rotary polisher speeds depending on the diamond grain size (see Table 4.3).

Sample polishing starts with a 30- or 15-μm diamond disk, depending on the material brittleness or roughness.

The tripod is placed on the diamond disk, and the polishing direction is as close as possible parallel to the sandwich glue line or layer interfaces.

Polishing is done by making regular movements on the disk, from the outside of the disk to the inside or vice versa, in order to prevent damaging the sample with polishing residues (Fig. 4.49). The tripod is held with one finger placed on the part near the L-bracket. This lets the operator feel any damage to or residual grains on the disk, and also to apply firm pressure on the sample, depending on its nature and the level of the polishing step.

When the whole disk surface has been used, it must be cleaned with lint-free paper soaked in water. Then spiral polishing is begun again.

Fig. 4.49 Spiral polishing from outside to inside the diamond wheel or vice versa

When the depth to be abraded (see Table 4.3) has been reached (i.e., no more gray traces visible on the diamond disk or a change in sensation under the fingers), this means that the material to be removed, determined by the retraction of the micrometer screw, has been abraded. Before moving on to the next abrasive paper, the micrometer screws must be retracted to the desired value, taking into account the diamond grain size used before (see Table 4.3).

Figures 4.50, 4.51, 4.52, and 4.53 show the surface state of the sample depending on the different gradings of diamond disks.

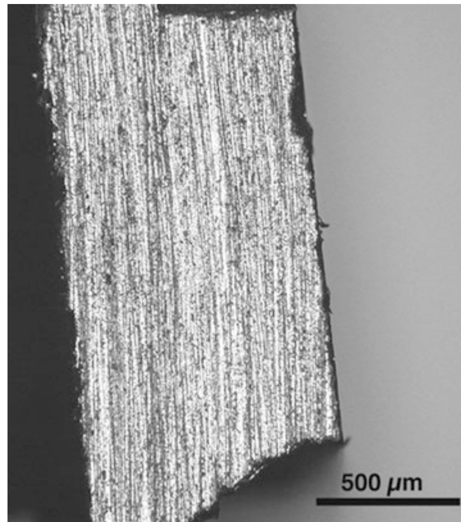

Fig. 4.50 Abrasion with a 15-μm diamond disk

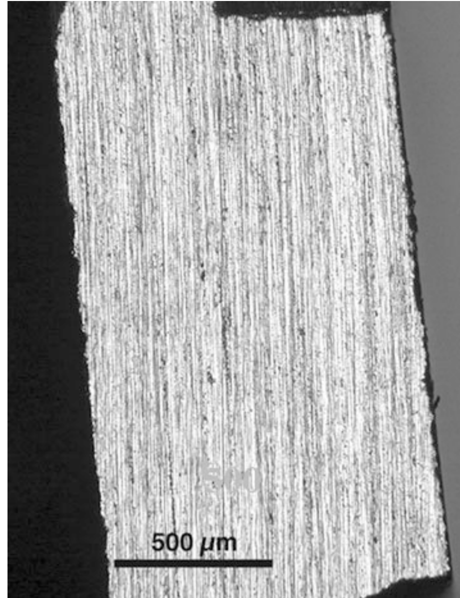

Fig. 4.51 Abrasion with a 6-μm diamond disk

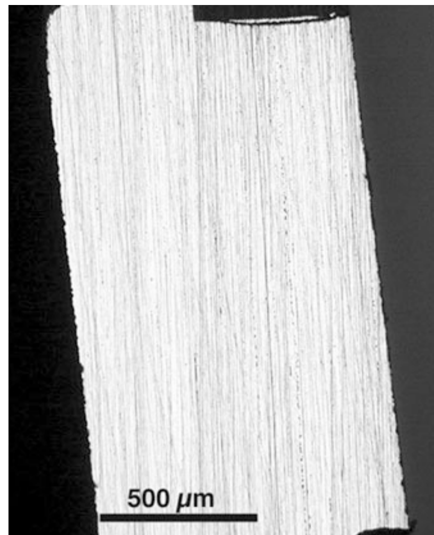

Fig. 4.52 Abrasion with a 1-μm diamond disk. The sandwich glue line becomes visible

Since the 0.5-μm diamond disk is very fragile, it is recommended to avoid rotation and polish using a straight-line movement parallel to the line of the sandwich glue (Fig. 4.54).

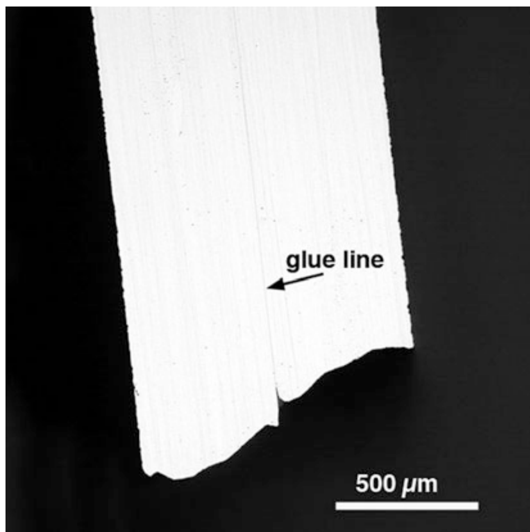

Fig. 4.53 Abrasion with a 0.5-μm diamond disk

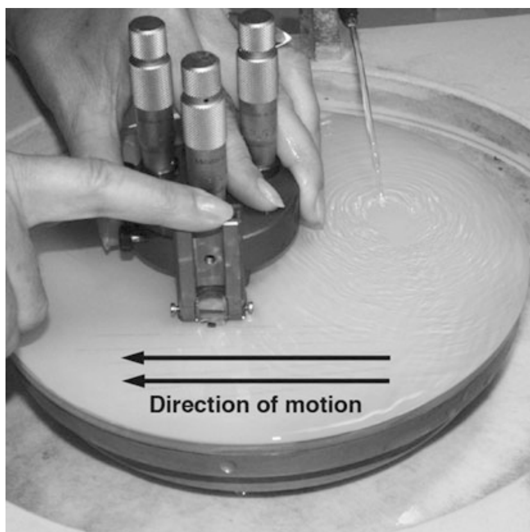

Fig. 4.54 Direction of motion when polishing with a 0.5-μm diamond disk

Final Polishing with Colloidal Silica or Other Media (Diamond, Alumina, etc.)

This polishing serves to remove any surface scratches and, for the second side, to further thin the sample or expand the spacing between the interference fringes (see 4.60*), resulting in a larger observable area.*

Polishing is done on a short-fiber soft felt, stuck to a perfectly flat support.

The felt is soaked with colloidal silica (alumina, diamond, etc.) diluted with distilled water. The polishing direction is *perpendicular* to the glue line so as to prevent the sharp edges of the sample from pulling up the felt and inducing damage to the material surface (Fig. 4.55). The average polishing time is 1–2 min.

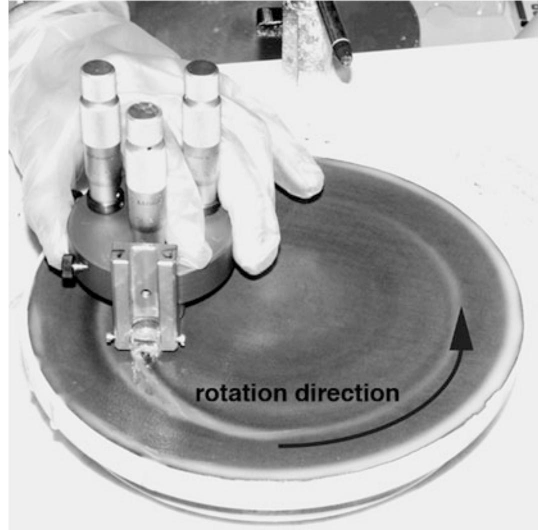

Fig. 4.55 Polishing with the colloid

Comment: Colloidal silica found on the market generally has a pH of 9.8. The result is a mechanical–chemical polishing that is not adapted to all types of materials (appearance of pitting or roughness on the sample surface, sputter, etc.). This is why it is necessary to use other very fine abrasives such as diamond, alumina.

Meticulous Cleaning of the Sample

The sample is held on the rotating felt while the colloid is removed by running rinse water on it and forcing the colloid out from the felt by hand (Fig. 4.56).

Fig. 4.56 Cleaning the sample and the felt

The felt must then be cleaned with distilled water before starting the polisher rotation and placing the sample back on the felt. Demineralized or distilled water must be added regularly. At this stage it is essential to dry the sample immediately with compressed air or CO_2 gas in order to prevent any residual traces from remaining on the surface.

Sample cleanliness and surface quality are checked using the “dark-field” mode of the optical microscope.

Ungluing the Sample and Sawing (If Necessary)

The tripod L-bracket is placed on the hot plate and the sample is unglued from the support.

The sample is then stuck using thermal glue to a support for the sawing step. Caution must be taken to protect the polished side using thermal glue.

The sample is sawed, preferably with a wire saw (Fig. 4.57) to a thickness of 500–800 μm, depending on the fragility of the material.

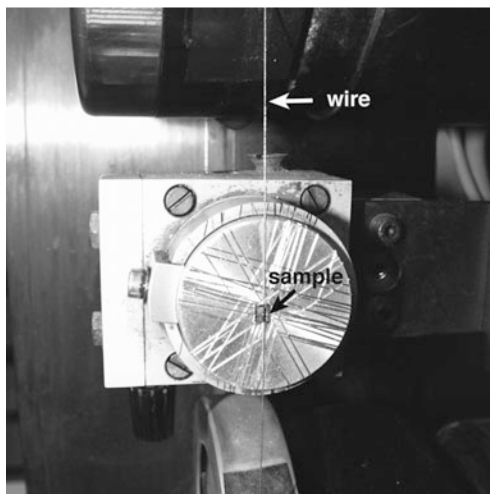

Fig. 4.57 The wire saw enables gentle cutting of the material. The wire diameter, abrasive grain size, and pressure can be selected based on the material brittleness

Plane Polishing of the Tripod’s Glass Support: Second Side Thinning

The glass support must be polished in order to ensure a level reference surface before introducing the wedge. The front micrometer screw of the tripod is fully retracted and will not be used afterward. The tripod is leveled by pressing on the glass and the two rear feet.

The glass support is polished until the entire surface of the glass is identically abraded (homogeneous surface roughness), using old diamond disks of 30, 15 μm, and then 6 μm.

Very important point: After the glass is polished, the angle should no longer be changed.

Gluing the Sample to the Support for Polishing the Second Side

The tripod's L-bracket is placed on the hot plate on its shorter length. A drop of thermal glue approximately 3.5 mm in diameter is placed on the glass cylinder, near the plane face (Fig. 4.46).

*Too much thermal glue can be a hindrance during polishing or can even distort measurements of sample thickness, which is performed as close as possible to the glue. Therefore it is necessary to make sure this drop of glue is no larger than 3.5 mm in diameter, i.e., a diameter larger than the support washer, in order to prevent any contact between the insoluble epoxy (used to glue the sample to the support washer) and the glass cylinder. In fact, in the case of a fragile or cleaved sample during preparation, it will be even better to glue the support washer to the sample when it is still stuck to the tripod L-bracket (see Section "*Mounting on the 3 mm Washer (2.3 mm)*" *3*).*

The sample is glued to the front side of the glass (Fig. 4.46).

The sample center must be pressed down using a wooden stick in order to produce as thin a glue layer as possible.

Introducing the Wedge Angle

The L-bracket is mounted onto the tripod, and then the two rear micrometer screws must be adjusted depending on the desired wedge angle (see Table 4.4). Lowering the rear feet between 300 and 500 μm is generally recommended.

Table 4.4 Lowering micrometer screws depending on the desired bevel angle and the thickness differential between the front and rear of a 1.5-mm-long sample, depending on the bevel angle

Wedge angle (°)	Micrometer screws drop (μm)	Difference in thickness between front and rear of a 1.5-mm-long sample (μm)
0.23	200	6
0.34	300	9
0.46	400	12
0.57	500	15
0.69	600	18

Polishing the Second Side

For the second side, measuring the sample thickness is very important and must be performed regularly. This measurement indicates when it is necessary to move to the next lower grade of diamond disk. It is done with an optical microscope by focusing on the thin edge of the sample, and then focusing on the glass support, as close as possible to the sample but outside the thermal glue area (Fig. 4.44).

Furthermore, it is necessary to take into account the thickness of the thermal glue used to glue the sample to the glass support. This thickness will depend on the type of thermal glue used. It can vary between 1 and 10 μm.

Polishing is performed *parallel* to the sandwich glue line, with the thinnest area being the trailing edge (Fig. 4.58).

Fig. 4.58 Direction of motion during second-side polishing. Angle exaggerated in figure

1. Polishing starts with a 30-μm diamond disk, down to a residual thickness of 170 μm *in front of the sample* (add in the glue thickness).
2. Polish with a 15-μm diamond disk down to a thickness of 80 μm.
3. After this step, it is necessary to verify that the right wedge angle has been introduced. This is done by measuring the difference in thickness between the front and rear of the sample (see Table 4.4). If the difference in thickness is not within the values indicated, the angle must be changed by retracting or lowering the micrometer screws.
4. Polish with a 6-μm diamond disk down to 30 μm.
5. Polish with a 3-μm diamond disk down to a residual thickness of approximately 10 μm. Some materials such as silicon will already present visible interference fringes (Figs. 4.59 and 4.60) on the edge of the wedge.
   At this stage, materials will not behave the same, depending on whether they are ductile or brittle, hard or soft.
6. Polish with a 1-μm diamond disk down to either the appearance of interference fringes along the edge of the sample (visible in different types of materials: Si, zircon, $LiTaO_3$, etc.) or until fragmentation or cleaving of the sample edge for a brittle material (GaAs, InP, some ceramics, etc.) or even until (slight) lifting of the edge for ductile materials (Cu, Al, etc.).
7. Polish using a 0.5-μm diamond disk until edge alignment is as homogeneous as possible, and then finish with a 0.1-μm diamond disk.

Final Polishing with Colloidal Silica or Another Abrasive

The polishing direction must be parallel to the glue line.

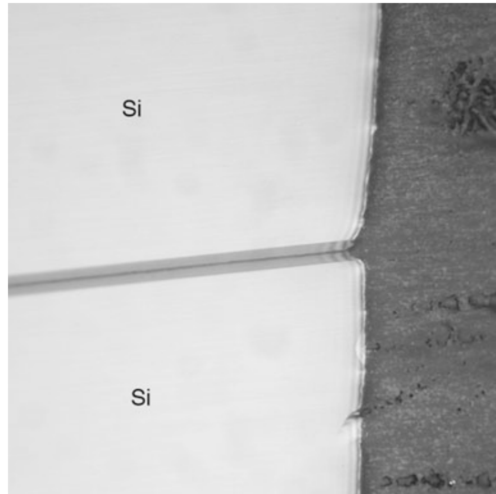

Fig. 4.59 Optical microscopy images, reflected light, of a cross section of a TiO_2/Si sample after final thinning with colloidal silica

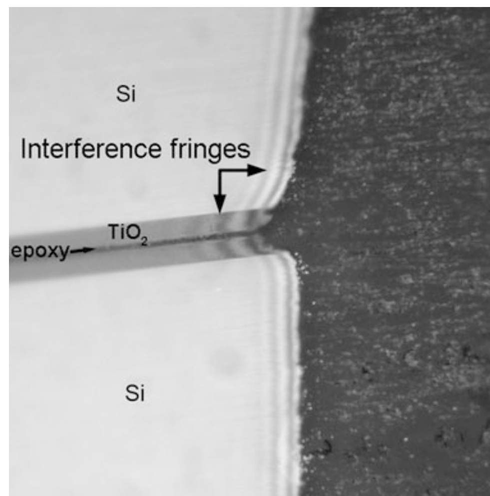

Fig. 4.60 Origin of interference fringes (or equal-thickness fringes): In episcopic illumination (incident light on the observation face), part of the light is reflected by the upper face of the sample and part passes through the sample and is then reflected by the lower face, then exiting the sample by the upper face. Since the speed of light is not the same in air and in the sample, both beams will interfere either destructively (d) ("canceling" or "extinguishing") or constructively (c) ("adding"), depending on the thickness of the slice and the wavelength of the light used. The corresponding mathematic relationships are $2nd$ cos= $m1_d$ and $2nd$ cos 1= $(1/2+m')1_c$, where n is the refractive index of the sample in air, m and m' are any two whole numbers (in practice, close because of the absorption in the slice and the losses of intensity to reflection). When illumination is normal to the surface of the slice, these relationships are reduced to $2nd= m1_d$ and $2nd= (1/2+m')1_c$. In white light, there are always one or two colors (two wavelengths, 1_d and 1_c) as the interference is either destructive or constructive, respectively, for a given thickness, d (except in parts too thin to satisfy these equations with m or m'=0)

Meticulous Cleaning of the Sample

The procedure is the same as the meticulous cleaning of the first side.

Mounting on the 3 mm Washer (2.3 mm)

There are two options for mounting the sample on a 3- or 2.3-mm washer adapted to the TEM:

(A) **Mounting the washer on a sample still glued to the glass cylinder**
This method is used in three situations:

- commonly used for any type of sample
- the sample was damaged or cleaved during preparation.
- to avoid leaving the sample in acetone too long (to prevent softening the glue at the interface)

For this method, it must be made absolutely certain that the thermal glue is spread over an area greater than the surface of the washer (3 or 2.3 mm) and that there are no holes in the glue that might let in the insoluble epoxy used to glue the washer.

Supplies

- Washer, 3 or 2.3 mm (Mo, Cu, Au, etc.)
- Smooth, stiff paper
- Razor blade
- Epoxy (Araldit Rapid, M Bond, G1)
- Eyebrow or similar hair (above all, very fine) mounted onto a manipulable support

Procedure
Cutting the washer:
Goal: Prevent ionic etching of the part of the washer that is to face the thin area of the sample. This pulverization would result in redeposition on the sample.

1. The washer must be kept flat by pressing on it with an object (stiff paper, glass slide, etc.) during handling. Cut the washer with a razor blade, leaving behind at least two-thirds so that it can be held stable in the TEM specimen holder (Fig. 4.61).
2. Under a stereoscopic microscope, a very small amount of glue is placed only on the thick part of the sample, e.g., using an eyebrow hair mounted onto a handle.
3. The washer is placed on the sample, as in the example shown in Fig. 4.41.
4. The glue is polymerized at room temperature for as long as possible, e.g., overnight. If this is not possible, the glue must be cured for at least an hour under a (UV) lamp.

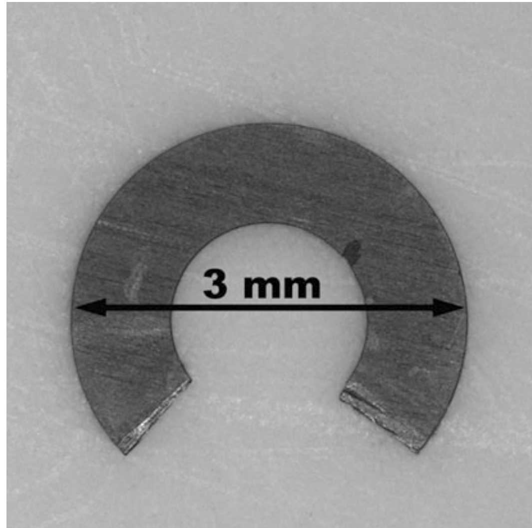

Fig. 4.61 Cut washer. The cutting edges might bend during the operation, so they must be flattened

5. Ungluing the sample: The tripod L-bracket is placed in a Petri dish containing filter paper and a wooden stick (toothpick) to raise the sample (Fig. 4.62). Pour in acetone and wait for the sample to come unglued.
6. Once the sample is unglued, the filter paper/sample still soaked with acetone is dunked directly in a bath of ethanol or another solvent. Perform this operation twice.

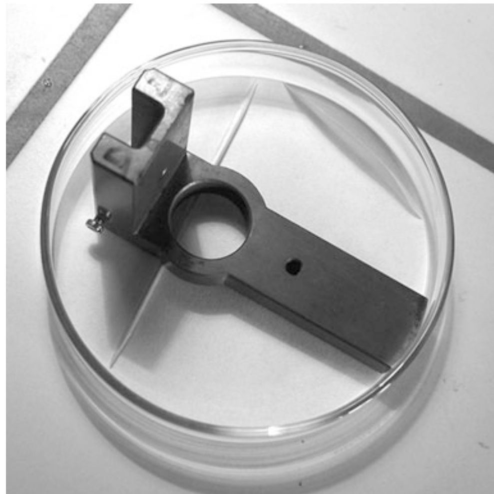

Fig. 4.62 L-bracket with mounted sample, pour in acetone

(B) **Mounting the sample on the washer**

1. Ungluing the sample: The procedure is the same as the one described above.

2. Cut the washer as in the first mounting method.
3. The washer is then held in a self-closing tweezer and a bit of glue is placed on the edge of the washer right where the sample is to be glued.
4. Place a very small drop of glue on the edge of the specimen holder slide (represented by a white dot in Fig. 4.63)

Fig. 4.63 Deposition of a drop of glue on the glass slide

5. The washer is placed on this small drop of glue, on the epoxy side, to glue the sample (represented by a white dot in Fig. 4.64).

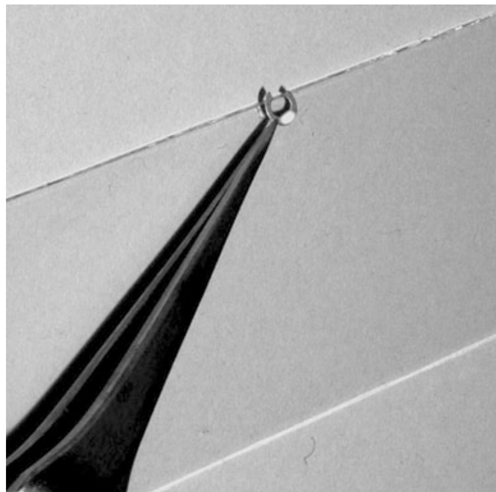

Fig. 4.64 The grid is put on the drop of glue

6. Under the stereoscopic microscope, use adhesive paper to firmly attach a filter paper, onto which the sample is placed (Fig. 4.65).

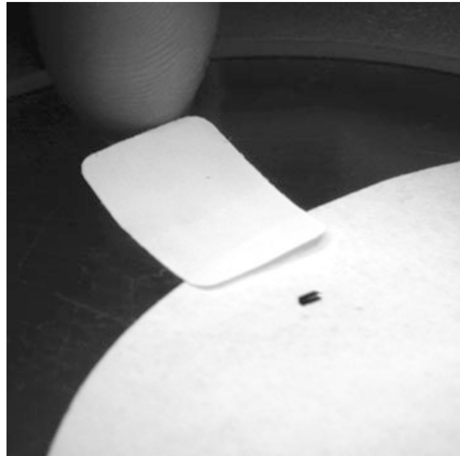

Fig. 4.65 Filter paper is stuck down to prevent moving

7. Take the specimen holder slide/washer setup, turn it over, and place it over the sample (Fig. 4.66). Slowly lower the setup until the washer touches the sample.

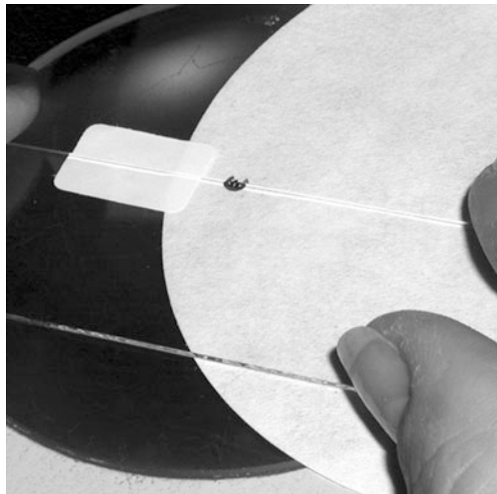

Fig. 4.66 The glass–sample setup is moved until the grid touches the sample

8. Turn it all over and slide it delicately onto a Teflon support (excess glue does not stick to Teflon) (Fig. 4.67).
   The entire setup should be heated for at least 10 min.

The sample is ready to be observed or ion milled to improve thinning.

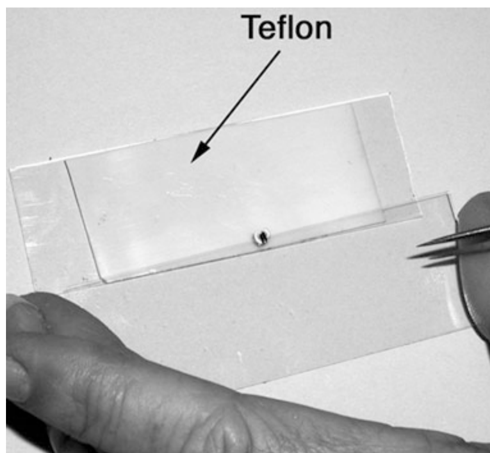

Fig. 4.67 The grid–sample setup is placed on a Teflon plate

Post-preparation: Ion-Milling Technique (Chapter 3, Section 5)

For a large number of materials (metals, some ceramics, mixed–composite materials, etc.), it will not be possible to achieve electron transparency through the wedge mechanical polishing, or sample may break (Figs. 4.68 and 4.69). Final thinning using the ion-milling technique will then be necessary in order to obtain an electron-transparent area (Figs. 4.69 and 4.70).

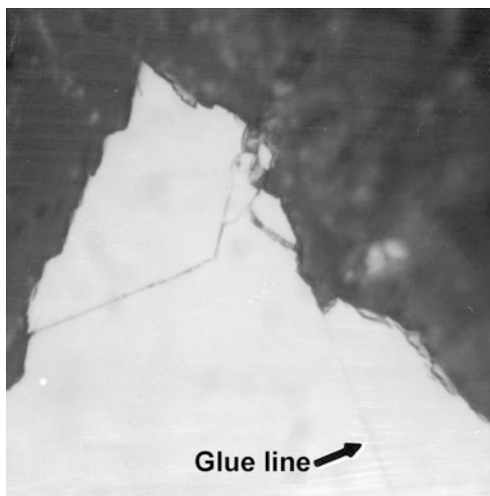

Fig. 4.68 Optical microscope image of a GaAs cross section. The *arrow* indicates the glue line. The sample broke during polishing

However, the tripod technique will have the advantage of reducing the area to be milled to a few microns thick, thereby reducing milling time, and consequently reducing the artifacts generated by this technique.

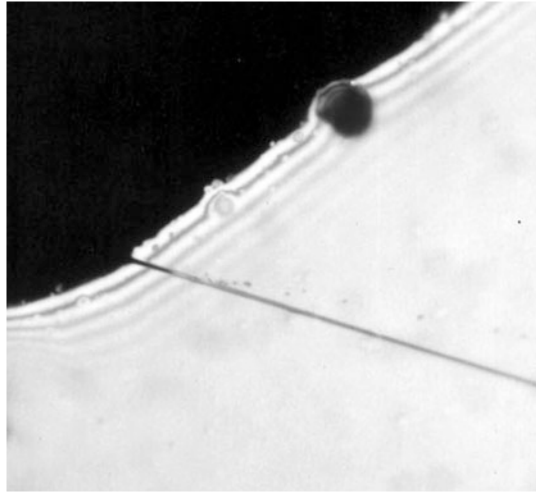

Fig. 4.69 Optical microscope image of the same sample after ion milling with the Gatan Duo-Mill: 1 h at 5 keV, 10 min at 2 keV, incidence angle 16°, two guns, full rotation (experimental conditions)

Ion Milling Conditions

Both sides of the sample are milled using single or double sectorial rotation without etching the thin area from the front, but from the rear or perpendicular to the sandwich glue line.

A gun angle as low as possible (between 1° and 7°) with regard to the sample plane and a low voltage (2 keV to 100 eV) will be selected in order to minimize irradiation damage induced by the ions.

Adapting the Tripod Method for Plane Polishing (Not Beveled) of a Cross Section

Some materials, e.g., very brittle materials, are not suited to the wedge preparation technique. In this case, the tripod is used to polish the sample "quickly," while getting a better polishing quality than in so-called "traditional" polishing (mounting the sample in a rigid support, polishing with SiC paper, diamond pastes, etc.).

Procedure: The procedure is identical in every way to the method described until Section "Introducing the Wedge Angle."

Modification: The front micrometer screw is retracted, the rear micrometer screws are lowered to the value of the desired residual thickness for the sample, plus the assumed thickness of the thermal glue (e.g., 20 μm + 10 μm of glue = 30 μm).

Polishing the second side is the same as polishing the first side, as described in the procedure outlined above. Sample levelness is verified by measuring both ends of the sample under the optical microscope.

Mounting on the washer: Do not cut the support washer.

Glue the washer to both ends of the sample (Fig. 4.70).

The sample is milled on both sides, perpendicular to the glue line, in double sectorial rotation.

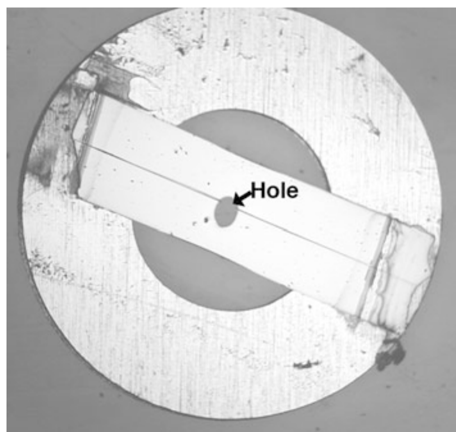

Fig. 4.70 Optical microscope image of a plane polished cross section, after double sectorial rotation ion milling, perpendicular to the sample glue line. The edges of the central hole are electron transparent

Adapting the Tripod Technique for a Beveled Plane Section

Advantages

The advantages are the same as with cross-sectional preparation. A large observable area is also produced (up to 2 mm).

Adapting the technique to prepare a bulk sample: The sample must be small enough to be mounted onto the washer (2 mm × 2 mm × 500 –700 μm, or half of a 3-mm disk).

Then proceed as with the bevel preparation of a cross section.

Adapting the technique for a multilayer sample (Fig. 4.71): Use the method given in Section "Plane Polishing of the Tripod's Glass Support: Second Side Thinning."

Glue the sample directly to the bottom of the glass, with the layers against the glue.

Follow the procedure given in Section "Polishing the Second Side."

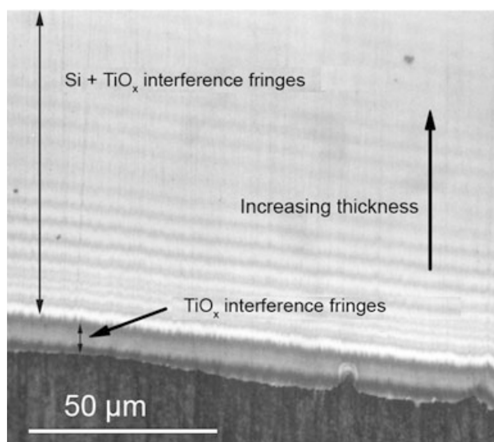

Fig. 4.71 Plane section of a TiO_x/Si [100] multilayer polished to electron transparency. Optical microscope, reflected light

If final thinning by ion milling is required, the sample is milled on just one side (substrate side) in single sector rotation, without etching the front of the thin area.

3.3 Variants

There are no variants.

3.4 Advantages

The method can be used to prepare a sample in any cutting orientation, longitudinal plane or cross section, producing large TEM-observable areas. The area to thin can be chosen precisely, down to the micrometric scale. Observation of the slice, first under an optical microscope then an electron microscope, helps to monitor the selected area at all scales. The technique does not induce chemical diffusion. There is no amorphization of surface layers (Figs. 4.72, 4.73, and 4.74).

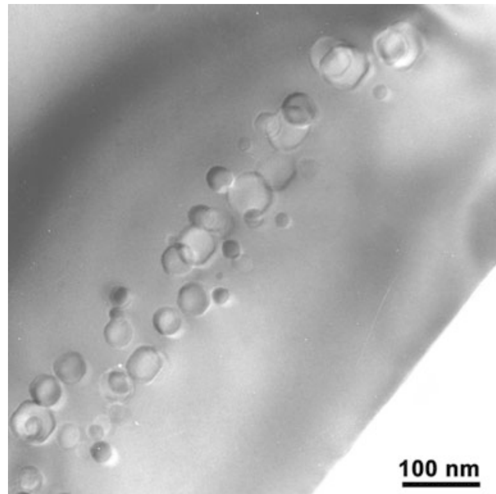

Fig. 4.72 Silicon sample with He implantation (cavities) under the surface. Bright-field TEM image (*J. Werckmann, IPCMS, Strasbourg*)

3.5 Limitations

The resulting thin slice is very brittle. It can be unstable and vibrate under the electron beam. This technique can introduce strain hardening in metallic samples. The interfaces can be etched by mechanical–chemical polishing.

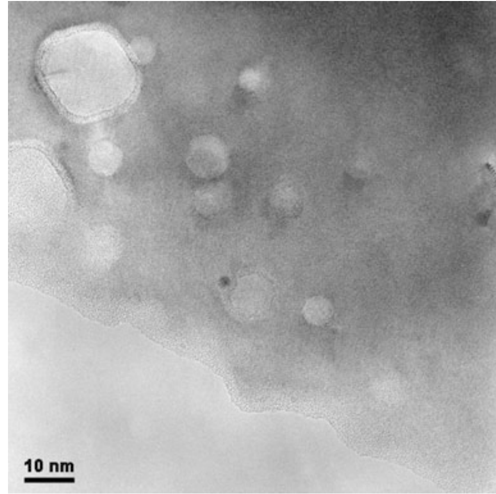

Fig. 4.73 Same sample as Fig. 4.72, thinner area, at higher magnification. Bright-field TEM image (*J. Werckmann, IPCMS, Strasbourg*)

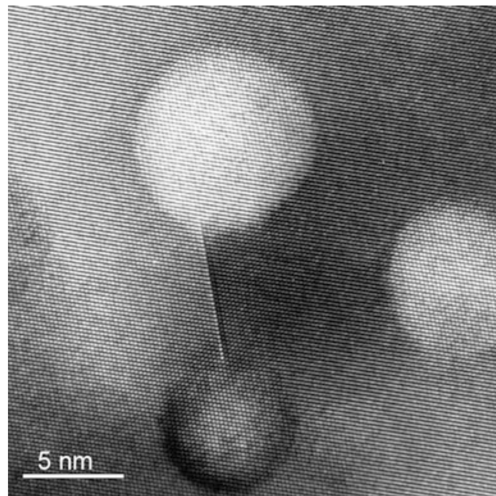

Fig. 4.74 HRTEM image of the same sample showing no surface amorphization (*J. Werckmann, IPCMS, Strasbourg*)

3.6 Artifacts

Observation can be hindered by colloidal polishing residues. The method can induce dislocations within the material. In some cases, preferential abrasion of the glue results in the loss of surface layers (Fig. 4.75).

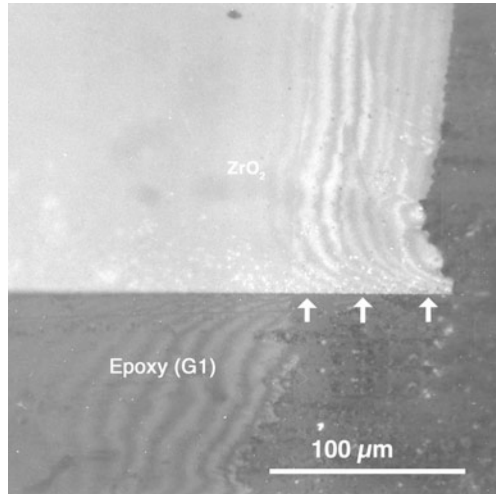

Fig. 4.75 Optical microscopic image of a sample of ZrO_2 in which particles have been implanted on the surface (*white dots*). In this example, the sample was not prepared in a sandwich. A glue film was spread on the surface of the material for cross-sectional wedge preparation. The glue wears away faster than the sample and does not protect the surface of the material (indicated by the *arrows*). The far surface is then abraded

3.7 Type of Analysis

Structure, crystal defects, crystallography, chemical composition and chemical bonds, properties.

3.8 Risks

Protection must be used against toxic vapors if solvents are used.

3.9 Conclusion

Tripod thinning is the technique of choice for hard, brittle, and multiphase materials. When compared with other ion-milling techniques, it results in a slice without amorphization and with large observable areas that are not possible to obtain with the FIB technique, for example.

4 Ultramicrotomy

4.1 Principle

Ultramicrotomy is a technique for producing ultrathin slices (30–100 nm) by making a micro-crack that progressively propagates into the sample, based on the areas of least resistance. The fracture is induced by the edge of a knife.

The hardness and plasticity of the sample are determinant factors. The material must be hard enough to produce the fracture without crushing and plastic enough so that the fracture spreads without breaking the sample into fragments during cutting.

This technique is performed using an instrument called an ultramicrotome, equipped with a glass or diamond knife. It is performed at room temperature and can only be carried out correctly on a small sample (0.1–1 mm^2 on average). Sections are easily obtained on a homogeneous sample of average hardness. If the sample is heterogeneous, it must be compact and the different phases of similar hardness should be next to one another.

If the sample is a powder or is too small to be held firmly, it will be resin-embedded; if it is porous, it will be infiltrated and embedded. A polymer resin is used to perform this embedding or infiltration. These resins are liquid at room temperature and they harden by polymerization.

If the sample is not hard enough to be cut at room temperature, it can be cut at low temperature (see Chapter 4, Section 5 on the Cryo-ultramicrotomy technique).

4.2 Operating Mode

4.2.1 Equipment and Supplies

The ultramicrotome consists of a specimen-holder assembly and a knife support, which allows for any orientation of the sample via specimen rotation/tilt. A goniometer system and 3D displacement movements of the knife provide this rotation and tilt. The ultramicrotome diagram in Fig. 4.76 shows the knife holder and different possible settings.

The sample is firmly attached to the arm (4.76a), which can move either manually or automatically. It follows a "D-shaped" trajectory, including a straight-line cutting movement in front of the knife and a lateral clearance. The sample is cut at a slow rate (4–0.1 mm/s); the clearance trajectory has a faster rate, with a very small retraction of the arm. At each passage, the arm undergoes an automatic piezoelectric advance, which is programmable between 30 and 200 nm.

Two advancement ranges are available: either between 30 and 150 nm, with increments of 1 nm, or between 150 nm and 2 μm, with increments of 10 nm.

The cutting and clearance speeds are independent of one other.

4.2.2 Procedure

The procedure consists of removing a small volume from the sample by machining it into a pyramidal shape, with a very small surface area at one end, and placing it into a special support. This assembly is then mounted on the microtome, against the knife, which is gently moved as close to the sample as possible. For ultra-thin sections, the knife is fixed and the specimen is advanced automatically, on a precise path with a very low cutting speed.

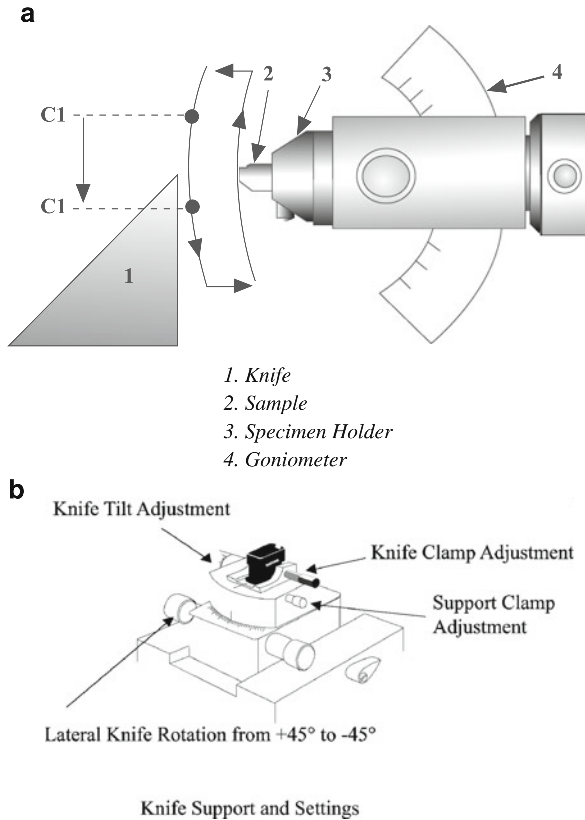

Fig. 4.76 (**a**) Microtome arm/specimen holder assembly and goniometer and (**b**) diagram of the knife settings

Inserting the Specimen into the Specimen Holder

The sample is firmly screwed into a sample holder before fixing it inside the goniometer (Fig. 4.77).

Rough bulk materials may be clamped directly between the teeth of the holder. Thin specimens can be clamped between two polystyrene faceplates before being inserted in the sample holder. Figure 4.77b shows a diagram of specimens clamped in the sample holder.

Choosing the Appropriate Knife

Depending on the cutting thicknesses required, as well as on the sample ductility and brittleness, a glass knife and/or a diamond knife can be used. Glass knives are produced with a 45° angle and diamond knives are manufactured with angles of 35° and 45° (Fig. 4.78).

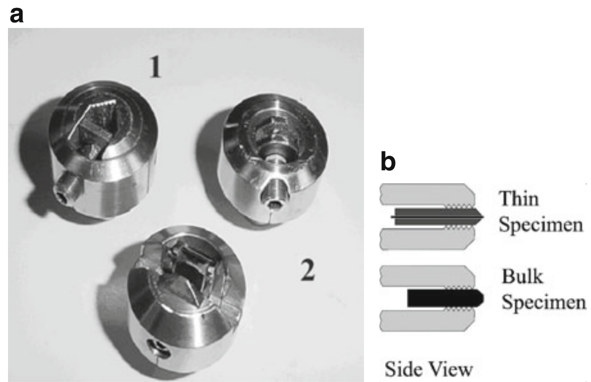

Fig. 4.77 (**a**) Different types of specimen holders: (1) specimen holder designed for a circular or cubic specimen and (2) specimen holders designed for flat specimens. (**b**) There is also a specimen holder for flat samples that does not have any teeth, and the thin specimen is locked between two small platelets without being damaged

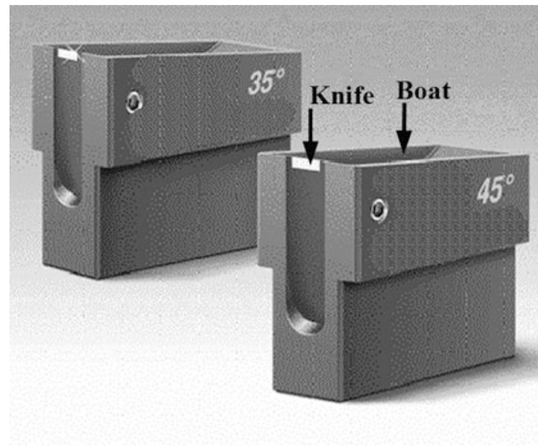

Fig. 4.78 Diamond knives, 35° and 45° angle

Knives are designed with a trough to be filled with water or other solution to allow for easier section collection. The sections will float on the liquid and can be easily collected. This works for material that does not dissolve in the usual solutions (water, DMSO, etc.). These sensitive samples must be "dry cut," and sections must be collected from the knife face, as explained in the cryo-ultramicrotomy technique (see Chapter 4, Section 5).

Knives with a 45° angle are adapted to the average hardness of epoxies used for embedding and are used for routine analyses. Knives with 35° angles minimize compression phenomena, and thus are better for producing thinner sections. They are becoming more widely used. This knife angle provides the best results for hard multiphased materials from solid-state physics. However, it is more fragile and wears out faster than a 45° angle knife. An oscillating knife can also be used and helps to reduce to almost zero compression for some soft biological or polymer materials (Fig. 4.80). This 35° angle diamond knife has a piezoelectric module mounted on the side, providing parallel oscillation of the knife at the desired amplitude (Fig. 4.79). The frequency can be set between 25 and 45 kHz, and the voltage

Fig. 4.79 Oscillating diamond knife

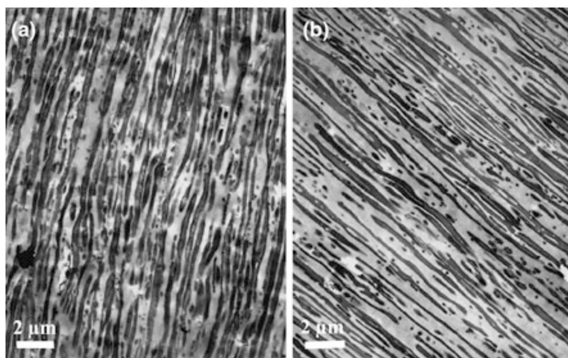

Fig. 4.80 TEM image of a polypropylene/ESI blend (**a**) sectioned at room temperature with a 35° diamond knife without oscillation. The waves are due to compression and (**b**) sectioned at room temperature with a 35° oscillating knife. The sample does not show any sign of compression (*Image courtesy of Dow Benelux BV and H. Gnaegi, Diatome, Bienne*)

can be set to between 0 and 30 V. The range of the section thickness is between 10 and 100 nm.

Mechanical Stresses Undergone by the Sample During Cutting

Sample compression may be reversible depending on the elasticity of the material. Compression is partially reduced by the spreading of water on the surface and by the effect of positive ions or vapors from organic solvents. However, hard or brittle samples undergoing excessive stresses will break. Samples that are too soft or plastic samples will crush under the knife edge and will not yield a slice. The diagram in Fig. 4.81 shows the compression exerted on the specimen and the reaction depending on the hardness. Chapter 4 of "Methodology" provides more explanation.

Knife Orientation and Water-Level Adjustment

The knife is placed on a support in the form of a cradle that can be oriented so as to slightly tilt the diamond knife edge, in order to have a clearing angle between 3° and 10°, often around 6°. This clearing angle depends on the hardness of the material.

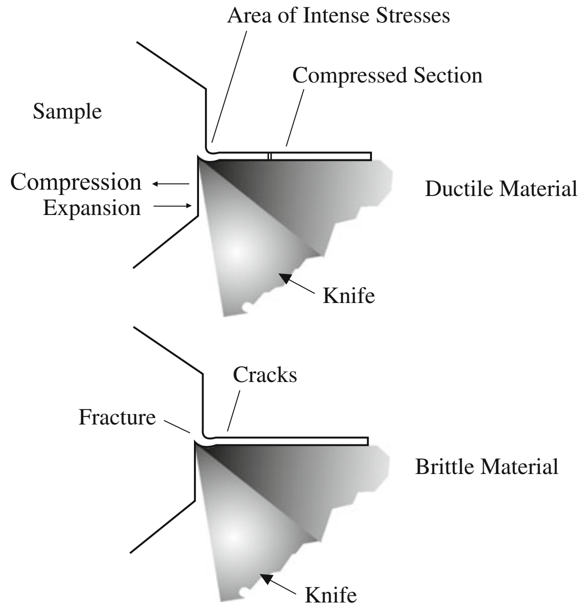

Fig. 4.81 Stresses undergone by a ductile or a brittle material during sectioning

Micrometer screws can be used to adjust the assembly both in depth and in laterally. Likewise, the assembly can be rotated in the cutting plane from –45° to +45°.

Usually the blade knife is equipped with a trough filled with very clean water, upon which the sections will spread out. The water level in the trough must be precisely adjusted so as to produce a low meniscus with the knife cutting edge. The sections spread out on the meniscus in the water, which reduces the compression for elastic materials due to the surface tension of the water.

Successive sections float on the surface of the water and thus can be easily picked up.

In some cases when there is risk of elements dissolving in water, the sections are made dry. The slices are more or less crushed on the knife cutting edge and are collected using an eyelash in order to be laid on the grid. Electrostatic charges that occur during cutting can be reduced by using an ionization device.

A stereoscopic microscope is used to align the sample with regard to the knife edge, as well as to make adjustments and monitor operations with great precision.

Sections are collected on a TEM grid either with or without a carbon film.

Selecting the Sectioning Conditions

As a general rule, for a hard sample a knife with an angle of 35°, a clearance angle of >5° and a slow cutting speed < 0.5 mm/s will be selected.

On the other hand, a 45° angle knife, a clearance angle of <5°, and a high cutting speed > 1 mm/s will be selected for a softer sample.

The section's "color," due to the refraction of light in the embedding resin, enables estimation of the section thickness. This depends on the refractive index of the resin, as well as on the incidence angle of the light reflected on the horizontal plane of the section. Usually, good slices are uniformly gray (50 nm) or golden (90 nm) in color. Under the right experimental conditions, the cutting speed must be adjusted so that sections are of the same color (transparent gray or bright golden), meaning they are of same thickness.

Preliminary Sample Preparations

Depending on the nature of the material to be cut, it may or may not have to undergo a preliminary treatment.

If the sample is bulk and compact, sawing is used to sample a cubic or parallelepipedic piece with sides measuring between 0.5 and 2 cm in length, which will be attached to the device's support provided for this purpose.

If the sample is porous, the air or water contained in the pores will be eliminated and replaced by a resin during infiltration.

The resins used for embedding and infiltration are the same. There are two types: epoxy resins or acrylic resins (see prior preparation in Chapter 2, Sections 8 and 9).

If the sample is hydrated, and it is living matter, then it is chemically unstable. First, chemical fixation is performed (see Chapter 2, Section 11) in order to be able to dehydrate the sample without too much damage to fine structures before proceeding with infiltration. The block made after polymerization will be treated as in the first case.

If the sample is a multilayer, it will be embedded in a resin after the material is cleaved and given a surface treatment for proper impregnation, so as to make a small fragment corresponding to the interface to be cut. It is embedded in a mold or a capsule. After the resin is polymerized using heat or ultraviolet light, the resulting block will then be treated like a bulk sample (see Chapter 2, Section 8).

If the sample is made of fine particles in the form of a powder or particles smaller than a millimeter, it will be embedded in resin in a mold or a capsule. In this case, a conical mold is preferred in order to minimize the sample quantity and especially to concentrate it near the pyramid of the embedding resin. After the resin is polymerized using heat or ultraviolet light, the resulting block will then be treated like a bulk sample (see Chapter 2, Section 8).

Trimming the Block: Producing a Pyramid

Cutting the sample to make a pyramid is carried out entirely under a stereoscopic microscope so as to cut the block without damaging the sample. In some cases, the sample must not be subjected to this preparation method, and this procedure should only be done with a glass or diamond knife.

Using a razor blade (or better still, a specialized tool), the far end of the sample is cut into a pyramid base a few millimeters tall. The cutting surface will be reduced to a square or rectangle with sides between 1 and 2 mm. The edges of this surface must be perfectly clean and smooth. Two sides must be strictly parallel to one another and the other two are square or trapezium.

The cut will be made by starting on one of the parallel sides of the pyramid. If the cutting surface is rectangular, the selected side should be a maximum of 1 mm. The lateral sides can be larger (Fig. 4.82a).

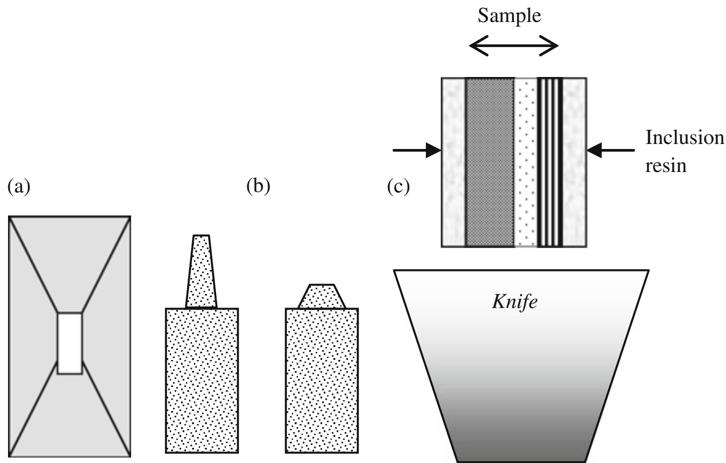

Fig. 4.82 Different views of specimen pyramid

The pyramid should not be too sharp in order to prevent vibration phenomena (Fig. 4.82b). It is better to prepare the sample a few hours in advance, allowing it to return to a stable state. Indeed, it has undergone stresses due to clamping in the support and to the preparation of the pyramid.

To prevent any damage to the sample, it is recommended to make a starting cut with a very fine razor blade. The subsequent shapings will be produced by making several semi-thin slices using the glass or diamond knife.

If a particular area must be chosen inside the sample, it is recommended to first make a fairly large cutting surface, e.g. 5 mm $\times$ 5 mm, and then rework the block after observation of a semi-thin slice (see below).

If the sample is multiphased, in the form of layers placed side-by-side next to one another, and especially if they are of a different hardnesses, it is recommended to plan the cutting direction in the direction of the layers in order to prevent the layers from crushing one another. In the worst case, the layers will separate from one another, which can be prevented by embedding the sample in a resin (Fig. 4.82c).

Relationship Between Block Surface Area and Section Thickness

The harder the sample, the smaller the cutting surface. Examples of the relationship between sample surface size and the thickness of the desired slice, for an average hardness sample, are presented in Table 4.5.

Table 4.5 Relationship between slice thickness and surface size of the block

Section thickness	Cutting surface size
1 μm	A few millimeters
100 nm	1 mm
80 nm	0.8 mm
70 nm	0.5 mm
60 nm	0.3 mm
50 nm	0.2 mm

Making Sections: Knife Adjustment

The block is attached to the device arm so as to make both of its sides parallel, and parallel to the knife cutting edge.

The knife is placed on its support, the cutting angle is selected (between 3° and 10°, often around 6°), and then it is firmly attached. The stereoscopic microscope must be adjusted in order to have proper stereoscopic vision by adjusting each ocular to its view as well as the distance between the two oculars based on the distance between one's eyes. The following operations are performed while looking into the microscope. The microscope must be focused on the knife cutting edge: Place the block in the field of vision and then gradually move the knife to the block.

Coarse Adjustment

The sample is moved toward the knife very delicately and in two movements: The coarse approach is made visually and is roughly adjusted by checking that the knife is far enough from the sample block, in front of the knife edge.

Fine Adjustment

This is made under the stereoscopic microscope. The knife and sample are placed together in the stereoscopic field of view, at low magnification, which helps to see what is going on at all times when the knife is moved toward the sample. The block and the knife must be close enough, but without touching, to make the following adjustments.

From this point on, it is necessary to work gradually, while avoiding any abrupt movements in order to completely adjust the parallel alignment between the whole surface of the block and the knife blade.

The different steps of the fine adjustment are represented in Fig. 4.83. By manually moving the block in front of the knife, we can view the verticality of the block

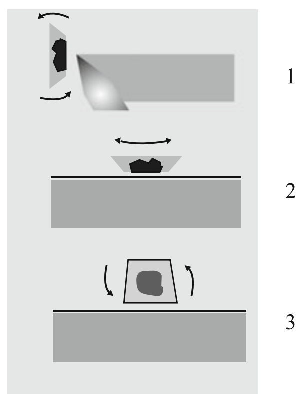

Fig. 4.83 Steps for moving the sample onto the diamond knife: 1, Side view, for judging the verticality of the block with regard to the knife; 2, Front view, for judging the alignment of the block with regard to the knife; and 3, adjusting the alignment

and the alignment between the block and the knife, which can then be adjusted using the micrometric movement of the specimen holder. The block is continually moved toward the knife by using the knife's micrometric advancement. When the block is close enough to the knife, there is a reflection of the knife on the block, as shown in Fig. 4.84. A reverse image with illumination below the knife is produced, enabling one to see the distance between the knife and the block, from the dark line that forms. The approach is completed by reducing the dark line as much as possible. This dark line must be as thin as possible over the entire surface of the pyramid being cut. A greater distance results in a thicker line. At this level, this dark line must be strictly aligned with the knife blade. Otherwise, it is aligned using the micrometric adjustment for rotating the knife. Finally, we are ready to begin cutting (Fig. 4.84).

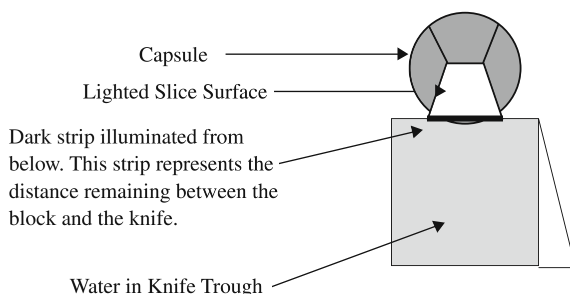

Fig. 4.84 Adjusting the fine approach of the sample block surface and knife

Then, a first slice, as thin as possible (approximately 0.5–1 μm), is made.

This is the most delicate part of cutting because we are very close to the knife, whose very brittle edge must not be damaged.

Using a syringe, the knife trough is filled so as to form a very white, shiny meniscus. This water level, up to the level of the knife edge, determines the proper production and spreading out of the slices. If there is too much water, the slices are

dragged off the back edge of the knife; if there is not enough, the slices spread out poorly and it is hard to see the thickness.

The cutting window, i.e., the slow-speed range, is adjusted with a device designed for this purpose on the ultramicrotome, and then the automatic movement of the block is begun. Cutting speed and thickness are selected. In the beginning, a few semi-fine sections can be made (0.5–1.5 μm) in order to produce a shiny, smooth cutting surface before moving on to thinner sections.

The semi-thin slices that are lifted up and deposited (or collected) on a glass slide can be observed under an optical microscope, which helps to localize the area of interest for the ultra-thin slice. The surface of the block can then be reduced in order to preserve this specific area.

Thin slices will be made in the form of a ribbon if both faces of the block are well aligned and parallel to the knife edge (Fig. 4.85).

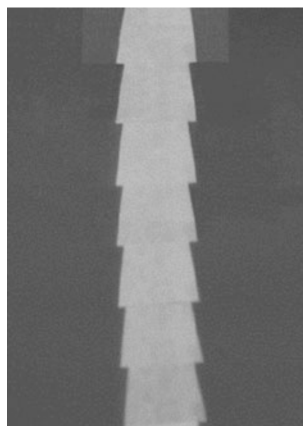

Fig. 4.85 Ribbon of sections on the surface of the water

If the slices fold, they can be spread out using chloroform or xylene vapor. It is also possible to reduce the electrostatic forces created by friction using a special ionization device.

Difficulties Encountered During Sectioning

In some cases, the sections do not remain stuck together in the form of a ribbon. The two parallel faces must simply be re-cut properly in order to correct this defect.

In other cases, the slices tend to be dragged off by the block and slide behind the knife blade. This defect could have different causes: the water level in the trough is too high, and it just needs to be corrected and verified that its reflection is metallic white; the lower edge of the block is not perfect and presents small burrs, and this just needs to be corrected using a razor blade; or if the block is improperly polymerized, it is hygroscopic and attracts the water from the trough. With each pass it gets wet and forms a droplet on the surface of the block. This phenomenon is common with polar acrylic resins. This block must be set aside and dried in a sterilizer

overnight at 310 or 333 K in order to complete polymerization. If the defect persists, a new embedding must be done.

Retrieval of Sections

Avoid touching the knife blade with the edge of the grid. To do this, an eyelash whose end is first dipped in the trough water is used; a few sections that have already been made are moved from the knife edge, and then they are recovered on a grid (see Fig. 4.86).

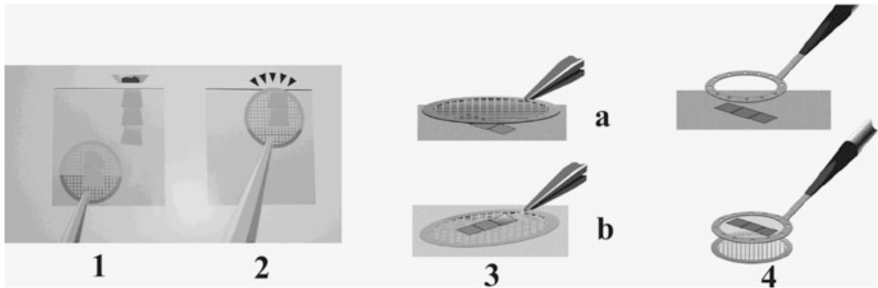

Fig. 4.86 Retrieving sections: 1, proper technique for slices far from the knife edge; 2, too close to the knife edge, risking damage; 3, section retrieval with a grid from above (a) or below (b); and 4, section retrieval with a special "perfect loop" tool

Three Methods Are Possible

First method: If the grid has a support film, the section ribbon is retrieved simply by touching the grid to the surface of the water where the sections are located.

Second method: If the grid is bare, the sections are recovered by dunking the grid into the water trough and the sections are gathered by delicately raising the grid out of the water, while keeping the ribbon of slices over the grid using an eyelash.

In both cases the excess water is removed by gently placing the grid on a filter paper.

Third method: The sections can be retrieved with a special "perfect loop" with a perforated perimeter. The sections are retrieved from above and then the water is removed using the corner of a filter paper.

4.2.3 Observations

Samples can be observed directly after a short drying time (e.g., overnight). Slices are often in the form of a ribbon that is checked at low magnification (Fig. 4.87) before making observations in TEM.

For sections deposited directly on a non-carbon-coated grid, if the material is insulating, the sample can be coated with a carbon layer by pulverizing a carbon

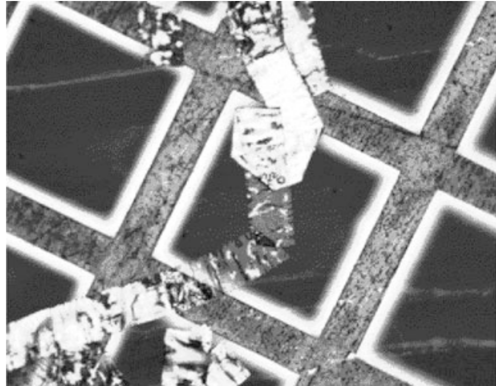

Fig. 4.87 View of a section ribbon deposited on the TEM grid (*H. Gnaegi, Diatome, Bienne, CH*)

braid. The conductive carbon layer helps to eliminate electrical charges visible by the instability induced on the sample during TEM observation.

If the structures to be observed (such as polymers or biological materials) do not have enough contrast under the electron beam, a treatment can be applied to fix heavy elements (osmium, lead, tungsten, ruthenium, uranium, etc.) onto certain structures. This treatment enhances the contrast difference and therefore improves observation (see Chapter 7, Section 3).

Figures 4.88 and 4.89 show muscle tissue in a cross-sectional and longitudinal plane after positive-staining contrast with uranyl acetate and lead citrate. These images show the importance of making cuts in specific directions when the material is textured. Here we see that the information provided is complementary, but requires having perfectly transversal or longitudinal cuts. These types of cuts are

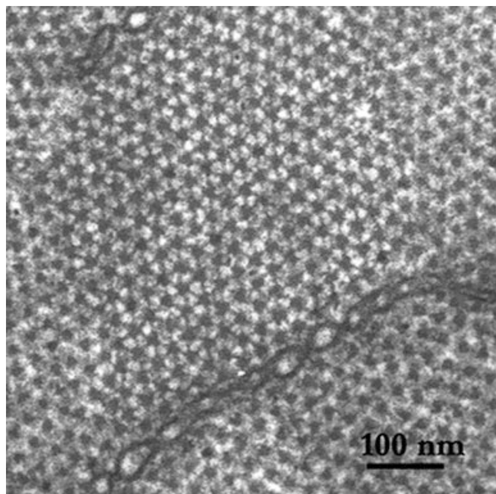

Fig. 4.88 Cross-sectional cut of myosin fibers in skeletal muscle. Chemical fixation, epoxy embedding, cutting, and positive-staining contrast with Ur/Pb (*J. Boumendil, CMEABG UCB-Lyon 1*)

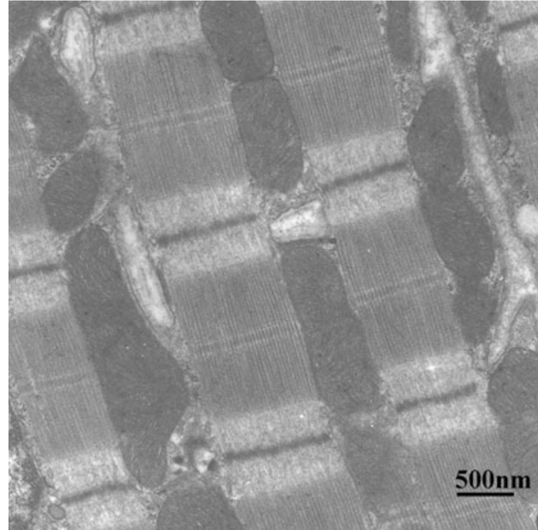

Fig. 4.89 Longitudinal cut of a striated muscle fiber. Alternation of myosin fibrils and actin and mitochondrial cords. Double chemical fixation, epoxy embedding, cutting, and positive-staining contrast (*J. Boumendil, CMEABG UCB-Lyon 1*)

possible when the sample is oriented during embedding and by using the different adjustments to the block and the diamond knife.

4.3 Variants

All sorts of variants are possible.

Each material represents an individual case and requires the technique to be adapted: knife angle selection (35°, 45°, or oscillating), cutting condition (knife tilt, cutting speed, etc.), and selection of slice retrieval mode.

4.4 Advantages

This technique is quite fast for a trained operator. It is used to produce many samples during one working session. The successive sections produced one after another can be used to make a 3D reconstruction of the structure.

This technique is used to select a specific area in the sample, at the optical microscopic scale if necessary. Sample orientation can be done in any direction, enabling the production of cross-sectional and longitudinal cuts (Figs. 4.88 and 4.89), which is indispensable if the material has an oriented texture. Through embedding, the same type of orientation can be made even if the sample is very small. Embedding also can be used for sectioning fine-particle samples of very small sizes (e.g., powder) (Fig. 4.90).

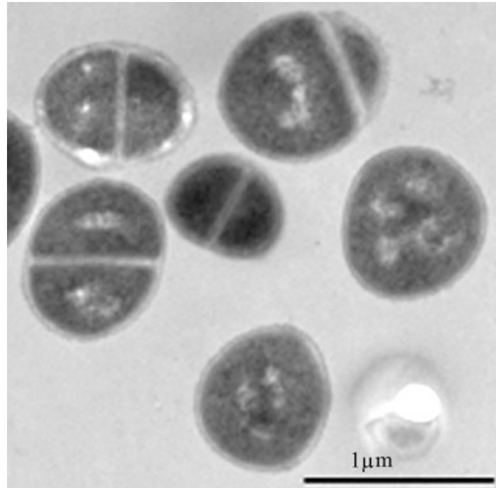

Fig. 4.90 *Staphylococcus aureus* bacteria, with double fixation, embedded in agar, dehydration, embedding in epoxy resin, cutting, and Ur/Pb contrast (*A. Rivoire, EZUS-UCB-Lyon 1*)

4.5 Limitations

This technique requires expensive equipment. It requires long training, precision, and constant working with the stereoscopic microscope, which is a significant source of fatigue.

The ultramicrotomy technique can be applied to all types of materials of average hardness and plasticity. It is based on the principle of rupture and generates many stresses depending on the hardness and brittleness of the material.

Mixed–composite samples of heterogeneous hardness risk becoming disorganized under the effect of the stresses, causing tearing, or in the opposite case, the crushing of some components.

Metallic samples will undergo surface strain hardening. Image interpretation requires vigilance.

4.6 Artifacts

Many artifacts are possible and must be detected.

Scratches on the cutting surface: These scratches appear in the cutting direction and are due to poor knife-blade quality.

Chatter: This results in undulations perpendicular to the cutting direction. They can have several causes: The sample is too soft, there is poor attachment of one of the parts of the device (knife or block), or the block pyramid is too sharp. These vibrations can appear in only some phases of the section, which results from a heterogeneity of the sample hardness (Figs. 4.91 and 4.92).

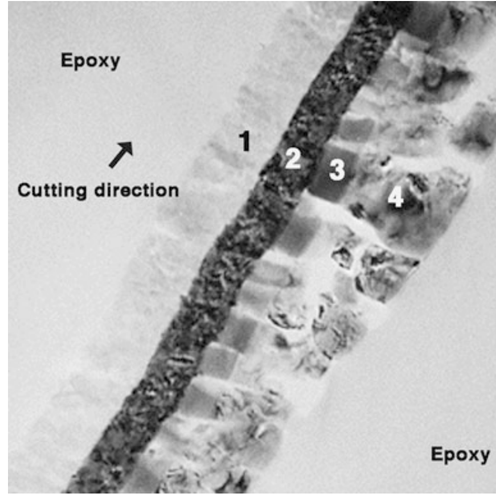

Fig. 4.91 A DCL(1)/Ti(2)/SiO_2(3)/Si(4) alloy multilayer embedded in epoxy resin, showing chatter and fractures in layers (3) and (4) (*H. Gnaegi, Diatome, Bienne*)

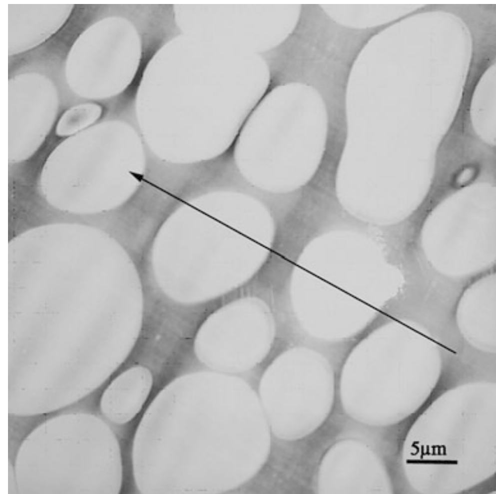

Fig. 4.92 Biphase polymer material. Chatter perpendicular to the cutting direction (*arrow*), resulting from compression (*stresses*), which does not disappear with the surface tension of the water where sections are draped (*A. Rivoire, EZUS-UCB, Lyon 1*)

In the extreme case, slices can compress on the knife edge if the block is very soft. Other cutting parameters can be tried: a knife angle from 48° to 50°, a cutting angle less than 5°, and a faster cutting speed. Cutting using cryo-ultramicrotomy may possibly resolve problems due to samples that are too soft.

In the case of multilayer materials of heterogeneous hardness (Fig. 4.93), there will be chatter or crushing phenomena. In order to prevent this phenomenon, the multilayer is embedded in a resin and cut by presenting the knife blade perpendicular to the layers (Fig. 4.82c). The goal of embedding is to prevent separation of the different layers.

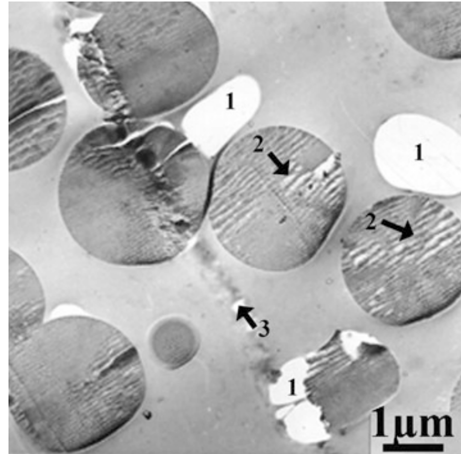

Fig. 4.93 Carbon mesophase particles embedded in epoxy resin showing holes (1), and chatter (2) and knife marks (3) (*J. Ayache, CNRS-UMR 8126- IGR, Villejuif*)

4.6.1 Examples of Defects

Tearing: In a heterogeneous material, harder inclusions can be pulled off the matrix, leaving a hole behind (Figs. 4.90, 4.93, and 4.94.)

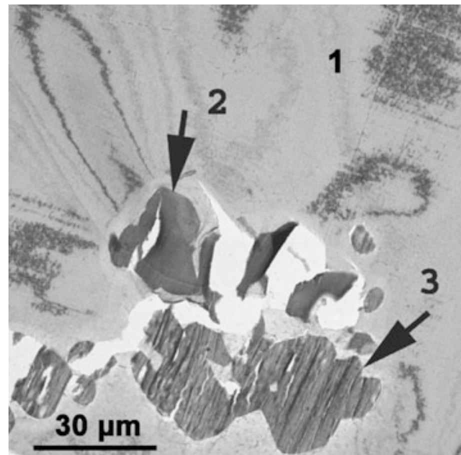

Fig. 4.94 Bulk aluminum-based alloy showing material tears in the section, formed due to mechanical behavior heterogeneity between the precipitates and the matrix. Precipitate 2, which is harder than precipitate 3, is expulsed from the cut. The hardness of precipitate 3 is greater than the matrix, but allows the interfaces with the matrix to persist (*J. Ayache, CNRS-UMR 8126- IGR, Villejuif*)

Material displacement: In a heterogeneous material, softer embedded objects can be displaced in the matrix upon which they are superimposed (Figs. 4.91 and 4.95).

Cracks in the section: The sample is too hard and too fragile for the cutting parameters (Fig. 4.91). We can try to use a knife with a more acute angle (30°), a larger cutting angle (5–8°), and/or a slower cutting speed.

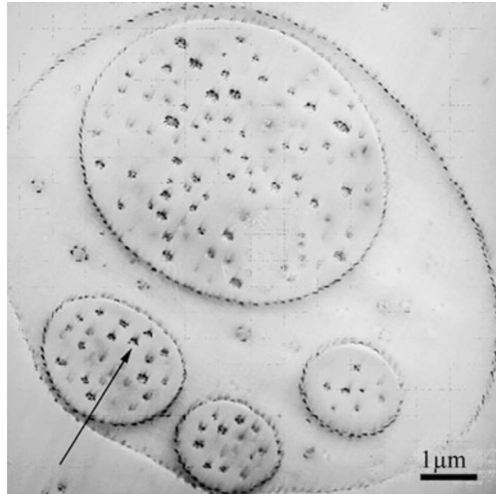

Fig. 4.95 Scratching of some overcharge particles in a polymer matrix. Deformation is in the cutting direction (*arrow*) (*A. Rivoire and E. Girard-Reydet, EZUS-UCB, Lyon 1*)

Irregular section thicknesses: These can result from poor infiltration–embedding or embedding quality. In the case of an un-embedded bulk sample (polymer), the sample might be too elastic. Cryo-ultramicrotomy may improve the hardness of the sample and reduce this problem.

Slice contamination by greasy traces or very fine dust. Under observation, these traces are superimposed on the structures and disrupt the image. Usually these traces come from the water in the cutting trough, due to working conditions that are not meticulous enough. In order to prevent this artifact, we use twice-distilled water and frequently degrease the instruments (tweezers, support grid, etching point, and eyelash) using alcohol.

The diamond knife edge must also always be cleaned after use in order to prevent cutting residues from sticking to the diamond edge, causing scratching (Fig. 4.93).

4.7 Type of Analysis

Structure, crystallography, chemical composition, chemical bonds, properties.

4.8 Risks

There are no risks.

4.9 Conclusion

This is the technique of choice for biological and polymer materials, either bulk or fine-particle, soft and/or multiphase materials. This technique is used to make ultra-thin sections of a constant 30- to 50-nm thickness, without amorphization, which

sets it apart from the FIB technique. When compared with the tripod technique and ion milling, it has the advantage of producing large observation areas (up to 1 mm), with constant thicknesses.

5 Cryo-ultramicrotomy

5.1 Principle

Cryo-ultramicrotomy uses the same basic principle as ultramicrotomy, but all of the preparations are made at a temperature below 273 K, enabling the production of thin sections (slices 50–150 nm). This technique is used for samples that are too soft to be cut at room temperature (polymers) and for hydrated materials. For polymers, lowering the temperature brings them near the glass-transition temperature and the material stiffens. For hydrated samples, lowering the temperature will transform water into ice that can be fractured by the ultramicrotome knife.

This technique is performed using a device called a cryo-ultramicrotome, equipped with a glass or diamond knife. It can only be carried out properly on a small surface area (0.1–0.5 mm^2). The technique is rather difficult to perform.

5.2 Operating Mode

5.2.1 Equipment and Supplies

The base ultramicrotomy equipment requires the addition of a special device containing a temperature-regulated enclosure, and a special knife support and specimen holder, which are both independently temperature-regulated. Temperature is regulated with an accuracy of nearly 0.5 K. The temperature range is between 273 and 153 K (Fig. 4.96).

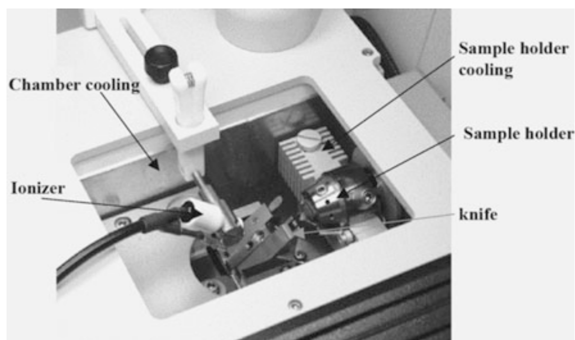

Fig. 4.96 Special chamber for cryo-ultramicrotomy

The device can also include a system for transferring the grid to the electron microscope. This system will keep the sample at a low temperature, in a dry

atmosphere, in order to prevent any frost contamination due to atmospheric water vapor.

The knife is usually a diamond knife without the water trough, because the cuts are made dry. In fact, it is not possible to use water because it freezes in the trough and the use of any other liquid is not recommended. Aromatic liquids that do not freeze at the temperatures in question do not have enough surface tension for the slices to float. Furthermore, these products are generally solvents that etch the sample.

Knives can have an angle of either 45°, 35°, or 25° (Fig. 4.97). The 25° knife is designed to cut frozen hydrated samples; this angle reduces compression and allows for good structural preservation. The cutting width of this knife is between 25 and 200 nm. A 35° knife will reduce the compression phenomena even further. In addition, an oscillating cryo-knife is available. This is a diamond knife on which a piezoelectric module provides oscillation parallel to the cutting edge, reducing compression effects.

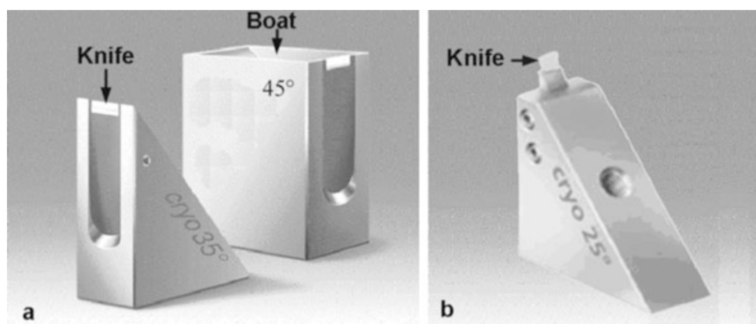

Fig. 4.97 **a** and **b** Diamond knives for cryo-ultramicrotomy: (**a**) 45 and 35° and (**b**) 25° angle, allowing least possible compression

5.2.2 Procedure

Preparing the Sample

The specimen can be a cube (1), a film (2), or a drop (Fig. 4.98), but it should always be very small in order to ensure proper freezing. The cutting surface area is extremely small, with sides measuring between 0.01 and 0.5 mm.

The specimen is glued to the support using glue that hardens as it cools.

The sample is cooled rapidly, either by dipping it in liquid nitrogen or slush nitrogen, or by cooling it gradually in the cryo-ultamicrotome chamber.

For polymers, the sample temperature must be stabilized just before reaching its glass-transition temperature (see “Methodology”, Chapter 1) in order to prevent

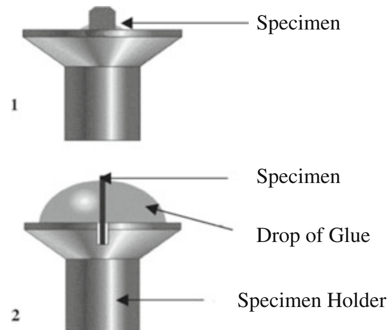

Fig. 4.98 Cold specimen holders

structural changes in the material. For a blend of polymers with different glass-transition temperatures, it is preferred to use the lowest temperature. However, this is not a hard and fast rule, and trial and error is often used to find the right temperature.

For biological samples, preparation can differ depending on whether the sample will be reheated and observed at room temperature or observed directly with a cooled specimen holder.

In the case of sections observed at room temperature, it is necessary to briefly fix the sample (see the section on variants in Chapter 2 , Section 11) and bathe it (from 1 h to overnight) in a cryoprotectant (glucose or sucrose in a saturated solution in water) before freezing it in slush nitrogen, following the Tokuyasu technique (1986).

Trimming the Block: Producing a Pyramid

If the sample will allow, sampling is made in a beveled shape so as to make a triangle. One of the angles of the triangle is placed facing the knife, which avoids having to make a pyramid. A few sections are removed before there is sufficient cutting surface area. This is often the case with polymers that are not too soft at room temperature.

For samples that are too soft before freezing, there is a special diamond knife available for preparing the cutting surface. The pyramid is made on the ultramicrotome under the stereoscopic microscope using this cooled tool, located next to the knife used for cutting the slices. Each side of the pyramid is made by making a 45° angle between the block and the knife and the four sides of the pyramid are made by rotating the specimen four times, a quarter turn each time.

Knife Orientation

The knife is placed on a support in the form of cradle that can be oriented so as to slightly tilt the knife in order to have a clearing angle between 3° and 10°, often around 6°.

Moving the Knife to the Sample

This method is the same as the one used for ultramicrotomy at room temperature.

The block is attached to the device arm so as to make both sides parallel, as well as parallel to the knife cutting edge.

The stereoscopic microscope must be adjusted in order to have proper stereoscopic vision by adjusting each ocular to its view. The following operations are performed while looking in the microscope. The microscope must be focused on the knife cutting edge: Place the sample in the field of vision and then gradually move the knife to the sample.

Coarse Adjustment

The knife is moved toward the sample very delicately and in two movements: The coarse approach is made visually and is adjusted by checking that the knife is far enough from the sample block.

Fine Adjustment

This is made under the stereoscopic microscope. The knife and sample are placed together in the stereoscopic field of view, which helps the operator to watch the advancement of the knife toward the sample. The block and the knife must be close enough, but without touching, in order to make the following adjustments.

From this point onwards, it is necessary to work very gradually, avoiding any abrupt movements. Since the block surface is very small, it is not necessary (or even possible) to make a precise approach before cutting, as in ultramicrotomy at room temperature. Indeed, there is no water in the cutting trough and therefore there is no reflection to guide the operator in making adjustments.

First, a few thick sections are made with the diamond knife designed to make the pyramid, until there is a sufficient cutting surface area. The knife is then changed by laterally moving its support, and then cutting can begin (Fig. 4.99a).

The cutting window, i.e., the slow-speed range, can be adjusted with a device designed for this purpose. Then we begin the automatic movement of the block and select the cutting speed and thickness.

Making and Retrieving Slices

Slices are collected dry, either one to one using an eyelash (Fig. 4.99b) or in a ribbon if it is possible to produce one.

If the sections are in a ribbon, we can slide it delicately onto the grid, which is usually without a support film (Fig. 4.100). Slices placed on the grid in this way adhere poorly to the mesh. In order to achieve better adherence, we press the slices on the grid either with a pestle or with another grid (Fig. 4.101). The grid is then removed from the cold chamber and returned to room temperature. It is sometimes coated with a plastic film (methyl cellulose) or a carbon film to protect it from

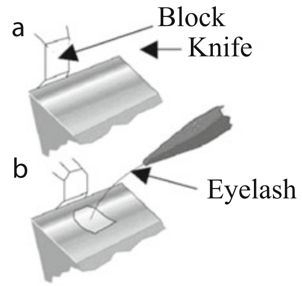

Fig. 4.99 Making a dry cut

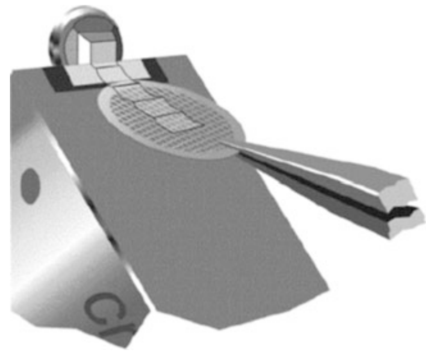

Fig. 4.100 Recovering dry sections

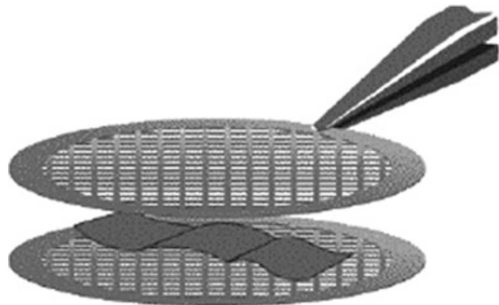

Fig. 4.101 Pressing sections to make them adhere to the grid

irradiation during observation. It can also be given additional contrast (see Chapter 7, Section 3).

Since cold sections are not gathered on the surface of a liquid, compression is even more significant than at room temperature and often hinders observation. In order to minimize this drawback, an ionizer is added during cutting in order to prevent sections from becoming electrostatically charged due to the rubbing of the knife on the block.

5.2.3 Observations

The sample sections can be observed directly (Fig. 4.102). If the structures do not have enough contrast under the electron beam, a treatment aimed at fixing heavy elements (osmium, lead, tungsten, ruthenium, uranium, etc.) onto certain structures can be applied. Observation will be done with a low-intensity electron beam, especially for biological samples that degrade very rapidly under beam irradiation.

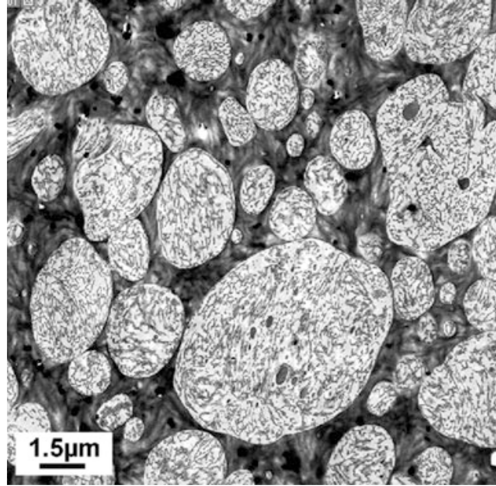

Fig. 4.102 Mixture of polyamide and PPE polymers (*H. Gnaegi, Diatome, Bienne*)

5.3 Variants

5.3.1 Tokuyasu Method

In biology, this type of section is used in order to proceed with immunolabeling or direct observation. In the example provided in Fig. 4.103, the tissue was fixed for 3 h in 3% paraformaldehyde, and then cryoprotected and frozen in liquid propane. Slices are made at 183 K for the block support temperature and 163 K for the knife temperature. Slices are picked up using a 2.3 M drop of sucrose (close to saturation), carried at the end of an eyelash or in a loop. In the cooled enclosure, the sucrose becomes slushy and sticky, making it possible to pick up the sections. When removed from the enclosure, the sections spread out on the drop of sucrose, which liquefies. The sections are placed on the grid, which is covered with a Formvar film, and then the sucrose is eliminated by placing the grid on a wet gelatin plate for 5 min. To prevent surface tension, immunolabeling is performed on plates of gelatin soaked in successively different reagents, rather than on drops, which could destroy the slices.

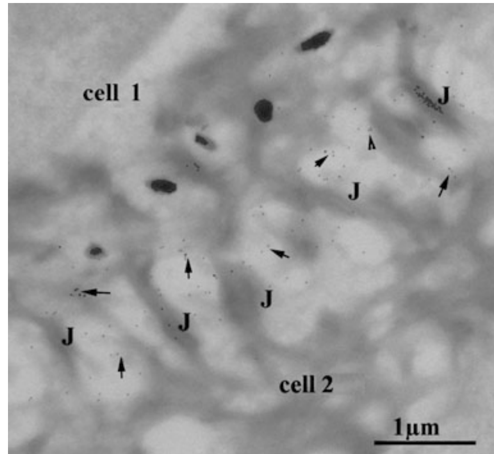

Fig. 4.103 Junction between two epidermal cells called keratinocytes. Immuno-localization of an epidermal proteoglycan located at the spike junctions between two cells. The gold labeling particles are 10 nm in size. J: intracellular junction; *arrows* point to areas of labeling (*M. Hafetek, EA3732, UCB-Lyon 1*)

5.4 Advantages

Cooling hardens materials that are too soft to be cut at room temperature. This technique is mainly used for polymers and composites containing soft polymers (Fig. 4.104).

It is because of the "Tokuyasu method" that this technique can be used in biology to immunolabel antigenic sites that may be lost during long fixation, dehydration, or embedding steps, at either room temperature or higher temperatures.

5.5 Limitations

Frozen preparations are very sensitive to the electron beam. Therefore, observation is carried out in low-dose mode in order to have a minimum intensity of the electron beam and prevent any degradation of the specimen.

This is a delicate technique to perform and it requires experience. Sections are often collected one by one and are difficult to remove from the knife edge. Compression problems are amplified compared to ultramicrotomy at room temperature, because there is no expansion of the slice due to the surface tension of the water in the knife trough for retrieving slices. It is difficult to transfer sections onto the grid because they are electrostatically charged, and they are pushed away from the copper or nickel grid. This problem is suppressed by the use of an ionizer.

The right cooling temperature must be found for polymers. For multiphase polymers, attention must be paid so as not to cause cracking in the material or tearing of harder areas and crushing of softer areas. The right temperature conditions are often within a few degrees and within minor variations in cutting speed. Slices are very brittle. They may be reinforced by depositing a carbon film onto the surface for chemical analysis, which requires an intense dose of electrons.

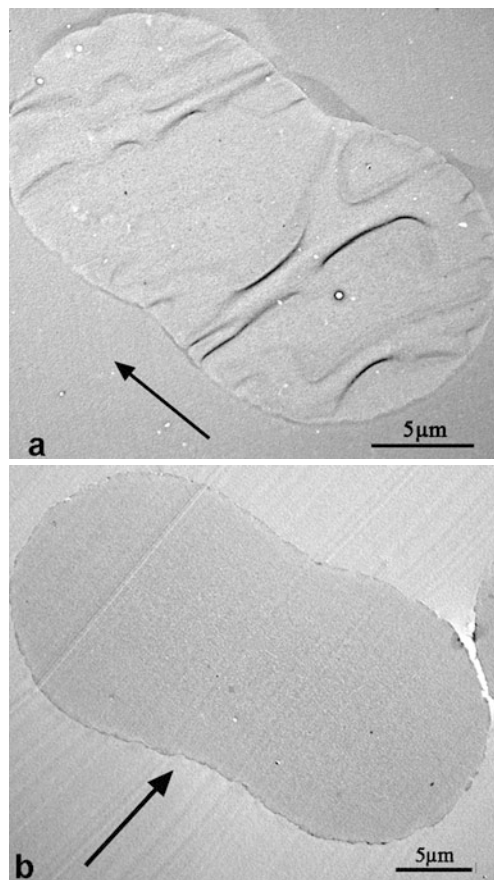

Fig. 4.104 (**a**) Copolymer cut at room temperature. The *arrow* indicates the cutting direction. (**b**) Same copolymer cut using cryo-ultramicrotomy. The *arrow* indicates the cutting direction. The polymer nodule, which is softer than the matrix, folds during cutting at room temperature, while it is perfectly spread out in cryo-ultramicrotomy. The *arrow* indicates the direction of the cut. Cold cutting was performed using a glass knife that left numerous scratches on the cutting surface. Scratches are parallel to the cutting direction (*A. Rivoire, EZUS- UCB Lyon 1*)

5.6 Artifacts

The artifacts produced by this technique are the same as those resulting from sectioning at room temperature: appearance of scratches parallel to the cutting direction and chatter perpendicular to the cutting direction. It is more common to have sections of irregular thickness and it is hard to get a section with each passage of the block in front of the knife. Furthermore, there is no light interference (observable in the resin when the slice floats on the surface of the water) in order to judge the thickness of the slices. Generally speaking, the sections will be thicker than those produced by ultramicrotomy and will often be thicker than 50 nm.

5.7 Type of Analysis

Structure, crystallography, chemical composition, chemical bonds, functional properties.

5.8 Risks

Risks related to the use of liquid nitrogen, particularly burns and anoxemia.

5.9 Conclusion

This technique is the only one available for preparing bulk and fine-particle biological and polymer materials that are too soft at room temperature. This helps to directly produce ultrathin slices with a constant thickness around 100 nm.

Bibliography

Al-Amoudi, A., Dubochet, J., Gnäegi, H., Lüthi, W., and Studer D. (2003). *J. Microsc.* **212**, 26–33.

Ayache, J. and Albarede, P. (1994). *ICEM 13*, **1**. Les Editions de Physique, Paris, 1023–1024.

Ayache, J. and Albarede, P. (1995). *Ultramicroscopy*, **60**, 195–206.

Benedict, J., Anderson, R., and Klepeis, S.J. (1992). *Specimen Preparation for Transmission Electron Microscopy of Materials-III, MRS Symposium Proceedings*, **254**. Bravman et al., Pittsburgh, PA, 121–140.

Benedicts, J.-P., et al. (1990). *Specimen Preparation for Transmission Electron Microscopy of Materials-II, MRS Symposium Proceedings*, **199.** Anderson et al., Pittsburgh, Pennsylvania, 189.

Bernhard, W. and Nancy, M.T. (1964). *J. Microsc. SFME*, **3**, 579–588.

Bonard, J.-M. and Ganière J.-D. (1996). *Ultramicroscopy*, **62**, 249–259.

Buffat, P.A., Ganière, J.-D., and Stadelmann, P. (1988). Nato Workshop, Bristol 12–17.9.

Cavalier, A., Spehner, D., and Humbel, B.M. (2008). *Handbook of Cryo -preparation Methods for Electron Microscopy*. CRC Press, Boca Rotan, FL.

Crang, R.F.E. and Klomparens, K.L. (1988). *Artifacts in Biological Electron Microscopy.* Plenum Press, New York, 65–79.

Ganière, J.D., Rheinhart, F.K., Spycher, R., Bourqui, B., Catana, A., Ruterana, P., Stadelmann, P., and Buffat, P.A. (1989). *J. Microsc. Spectrosc. Electron.*, **14**, 407–414.

Glaisher, R.W. and Cockayne, D.J.H. (1993). *Micron*, **24**(3), 257–264.

Golmayo, D., Dotor, M.L., and Quintana, C. (2003). *J. Cryst. Growth*, **253**, 167–173.

Griffiths, G., Mc Dowell, A., Black, R., and Dubochet, J. (1984). *J. Ultrastruct. Res.*, **89**, 65–78.

Hsieh, C.E., Marko, M., Frank, J., and Mannella, C.A. (2001). *J. Struct. Biol.*, **153**, 25–32.

Jesior, J.C. (1986). *J. Ultrastruct. Res. Mol. Struct.*, **95**, 210–217.

Jesior, J.C. (1989). *Scanning Microsc. Suppl.*, **3**, 147–153.

Kakibayashi, H. and Nagata, F. (1985). *Jpn. J. Appl. Phys.*, **24**(12), L905–L907.

Klepeis, S.J. et al. (1988). *Specimen Preparation for Transmission Electron Microscopy of Materials.* MRS Symposium Proceedings, vol. 115. Bravman et al., Pittsburgh, PA, 179.

Mc Caffrey, J.-P. (1991). *Ultramicroscopy*, **38**, 149–157.

McIntosh J.R. (2001). *J. Cell. Biol.*, **153**, 25–32.

Michel, M., Gnäegi, H., and Muller, M. (1992). *J. Microsc.*, **166**, 43–56.

Quintana, C., Menéndez, J.L., Huttel, Y., Lancin, M., Navarro, E., and Cebollada, A. (2003). *Thin Solid Films*, **434**, 228–238.

Quintana, C. (1997). *Micron*, **28**(3), 217–219.

Reid, N. and Beesley, J.E. (1991). *Sectioning and Cryosectioning for Electron Microscopy in Practical Methods in Electron Microscopy*, vol. 13 (ed. A.M. Glauert). Elsevier, Amsterdam, 1–245.

Richter, K., Gnäegi, H., and Dubochet, J. (1991). *J. Microsc.*, **163**, 19–28.

Robards, A.W. and Sleyter, U.B. (1985). *Low Temperature Methods in Biological Electron Microscopy, Freezing-Sectioning*. Elsevier, Amsterdam, 201–242.

Sitte, H. (1996). *Rev. Scanning Microsc. Suppl.*, **10**, 87–466.

Studer, D. and Gnäegi, H. (2000). *J. Microsc.*, **197**, 94–100.

Tokuyasu, K.T. (1986). *J. Microsc.*, **143**, 139–150.

Treaholt, C., Wen, J.G., Sventchnikov, V., Delsing, A., and Zandbergen, H.W. (1993). *Physica C*, **206**, 318–328.

Vastenhout, J.S. and Gnäegi, H. (2002). *Microsc. Microanal.*, **8**(2).