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Sample Preparation Handbook for Transmission Electron Microscopy

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Foreword by Ron Anderson

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TEM image of freezing defects in a frozen thin film, showing clusters of segregated crystals along the holes of the carbon membrane. (Baconnais S., CNRS-UMR8126, Villejuif, FR).

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When faced with a choice,
Regardless of the path you choose,
Don't go down it halfway.
Be patient, be persistent,
Be courageous, and creative.
The reward is at the end....

Jeanne Ayache

Foreword

Successful transmission electron microscopy in all of its manifestations depends on the quality of the specimens examined. Biological specimen preparation protocols have usually been more rigorous and time consuming than those in the physical sciences. For this reason, there has been a wealth of scientific literature detailing specific preparation steps and numerous excellent books on the preparation of biological thin specimens. This does not mean to imply that physical science specimen preparation is trivial. For the most part, most physical science thin specimen preparation protocols can be executed in a matter of a few hours using straightforward steps. Over the years, there has been a steady stream of papers written on various aspects of preparing thin specimens from bulk materials. However, aside from several seminal textbooks and a series of book compilations produced by the Material Research Society in the 1990s, no recent comprehensive books on thin specimen preparation have appeared until this present work, first in French and now in English.

Everyone knows that the data needed to solve a problem quickly are more important than ever. A modern TEM laboratory with supporting SEMs, light microscopes, analytical spectrometers, computers, and specimen preparation equipment is an investment of several million US dollars. Fifty years ago, electropolishing, chemical polishing, and replication methods were the principal specimen preparation methods. Ion milling, tripod polishing, and focused ion beam (FIB) tool methods were yet to be introduced. Today, a modern ion milling tool can cost tens of thousands of dollars and a fully outfitted FIB tool can easily cost a million dollars. With investments of this magnitude – made necessary by the demands placed on modern TEM analysis – it is paramount that the staff preparing TEM specimens have all of the training and resources possible to carry out their duties. This is where the book in your hands comes in!

But thin specimen preparation is more than just laboratory hardware and excellent protocols for thinning a specimen to electron transparency. Successful thin specimen preparation also requires knowledge of the information required from the TEM analysis. The question determines the method! For example, there may be several methods that could be used to produce specimens of the same material, but without a clear idea of the information required, even successfully thinned specimens may be only marginally useful. Thus, considerable thought should go into understanding the problem. In some cases, information from light microscopy,

SEM, powder X-ray diffraction, and a trip to the library (or at least to the Internet) will solve the problem without even making a TEM thin specimen. In other cases, such information will be helpful not only in the TEM analysis itself but also in preparing appropriate thin specimens for such analysis. Unlike analytical methods that routinely deal with completely unknown specimens, say powder XRD, successful TEM requires the analyst to bring considerable knowledge to the microscope – and even to the specimen preparation! It is more important to bring knowledge to the specimen prep. Wrong prep, and the scope time is useless. Thus, we should set some realistic goals for our thin specimens and bring as much intelligence to the table as possible. Here are three goals to consider no matter what material is to be thinned:

Goal 1: *To produce an electron transparent specimen representative of the bulk material in both structure and composition.* To meet this requirement, the researcher must have a comprehensive knowledge of the structure of the material system to be studied. It might be possible to produce an electron transparent specimen by beating your material with a hammer and collecting the thinnest shards for observation. However, it is likely that the resulting specimen would not have any relationship to the structure of the material before it was so "processed." The writer is certain that there are researchers working with silicon semiconductor specimens who think that the microstructure of single crystal Si contains numerous "salt and pepper" small defects that are actually ion milling artifacts. A good rule of thumb to follow is to prepare TEM specimens by more than one method if possible. Comparing ion-milled Si with chemically polished Si thin sections will immediately establish the true microstructure of Si, for example. The well-prepared analyst should know that as-grown single-crystal Si should be featureless and that an Al–Cu alloy will contain a family of precipitates as a function of the specimen's thermal history. Facts like these on any system to be studied may be found in the literature or learned in discussion with colleagues. This handbook provides clear instruction on the many specimen preparation methods by which it should be possible to produce alternative studies of a given material – with the advantages, disadvantages, and artifact risks of each – so that an analyst can deduce the true microstructure of their specimen.

Goal 2: *To provide easy access to the required specimen information.* This would not be a problem if the specimen preparation protocol always yielded "ideal" specimens. What is an "ideal" specimen? First, the transmission electron microscopy specimen must be thin. How thin? Optimum thickness varies with the microscopy application and the information desired. The optimum thickness for dislocation density measurements may be 100 nm or greater, but the optimum thickness for electron energy loss spectrometry measurements is often less than 10 nm. The ideal specimen should maintain an ideal thickness over a large area. Second, an ideal specimen should be flat, strong, homogenous, and stable under the electron beam for hours and in the laboratory environment for years. Finally, an ideal specimen should be clean, conducting, and non-magnetic. The reader may well conclude that there is no such thing as an ideal specimen. Compromises have to be made. Perhaps no single specimen preparation method is perfect. Given a thin film alloy containing precipitates, for example, electropolishing might thin the alloy matrix but leave the precipitates

too thick to analyze, whereas, ion milling might thin the precipitates but induce objectionable artifacts in the film matrix. Specimen preparation may also be limited by external factors. In the example just given, a focused ion beam (FIB) tool could prepare a satisfactory thin specimen exhibiting both the precipitates and the matrix. However, such a tool can be very expensive, and the analyst's laboratory may not have access to one. Thus, less-expensive methods must be found. Expertise in as many thin specimen preparation protocols as possible is a great advantage in any laboratory, hence the utility of the present handbook.

Goal 3: *To produce a thin specimen that enables the microstructure of the material to be accurately studied and convincingly illustrated in reports and peer-reviewed publications.* The end goal of thin specimen preparation is the production of new knowledge displayed as micrographs in publications. Correct, artifact-free exposition of the specimen microstructure is all that matters in the final analysis and will probably be the only thing recognized by the scientific community. That community, and the analyst's management, really will not care which or how many preparation protocols are employed. It is the artistic skill and the knowledge of the specimen preparer that counts, hence the value of the present handbook.

This book provides the novice with a grounding in the major specimen preparation methods in use today, assessing their merits, and identifying those modalities that are most likely to yield success. Experienced specimen preparers can use these protocols to find alternative ways to prepare their standard specimens. In addition, new requirements may become necessary, such as high-spatial resolution in the prepared thin specimen itself, where the locations of specific predetermined sites are required to be within 100 nm. Moreover, now it is often required to prepare thin specimens in much shorter times than a decade ago.

For the most part, this handbook serves the physical science community. However, there has been a trend in recent years for performing materials science analysis in biological laboratories – especially with the increase in work on biomaterials and biomimetics. So what do biologists do with materials samples? Where do they turn for specimen preparation help? I am suggesting that this book and web site are the place.

The authors have chosen a unique format for publishing their work. They originally considered a book in two volumes with a companion CD. This static approach, where readers would wait between editions to learn new content, was abandoned in favor of a handbook with a companion dynamic web site, where the content can be updated as soon as new material appears. As fully explained in this handbook, the researcher is provided with web-based guides containing both a database of materials and an "automated route" to lead to the most appropriate specimen preparation technique based on sample properties and the choice of microscopy technique. The web content is extended via links to international microscopy centers and databases. The short files on the web site are augmented by the extensive treatment each topic receives in the book. You, the reader, can be part of this novel pedagogical approach; there are facilities whereby you may add updates and new content to the web site as you develop them. Manufacturers making specimen preparation tools and supplies

may also contribute to the project. This remarkable work will remain current and provide continually increasing value to the specimen preparation community.

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Fellow of the Microscopy Society of America and Past President
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September 2009

Preface to the English Edition

It was a real adventure for our special club of five. Jeanne Ayache selected four collaborators for our supposed expertise in different areas of sample preparation and our belief that we really owed this "little job" the preparation of a guide to sample preparation, to our young and new colleagues. It is always attractive to share the experience of a career, and anyway the project (we thought!) could be completed within a year. Five microscopy specialists, each working in a different discipline and having a long-standing practice of teaching courses in this field, constituted a one-of-a-kind team.

With 5-times-20 years of experience, which, as they say in finance, comes to 100 years in accumulated surplus, our collaboration could not be reduced to a little 200-page manual. As the meetings went by, the program took shape, not without pains, resulting in a web site (in French and English) and the volume that we offer you today.

The first difficulty of this project was the language. Although we all speak French, we very quickly came up against our personal jargon: the "dialects" of a lab or of a scientific community (physicist, biologist, chemist, etc.). The richness of the French language is such that translations from French into English are different from one field to another, and habits are thrown in. For example, physicists talk about microstructures down to the scale of the nanometer, while biologists talk about ultrastructures and often stop at the scale of a tenth of a micron. Biologists who practically perform nothing but ultramicrotomy talk of "cuts," while physicists prepare "thin slices," even when they are making cuts! It almost felt like being in the tower of Babel. In short, we first had to create a glossary with a definition that provides exactly the meaning ascribed to the word used. This was a task that called for many debates and all our energy during long meetings. Once this primordial step in any interdisciplinary or cross-disciplinary undertaking was completed, everyone drafted the sections on techniques they practice frequently and know well.

The second unique aspect of the project was the collective reading of the various techniques, always with an "uninitiated" member in the group who knew nothing about the field being introduced. How should you explain an electrochemical manipulation to a biologist and an immunolabeling to a metallurgist, for example? The result is a selection of expressions accessible to all, including the non-specialist, at the expense of a super-precise aspect, of course. The techniques that we present

here are written so that they may be understood by those who have never practiced them. We not only give you the outlines that make it possible to understand their implementation, their limits, and their artifacts, but also often present the details that enable their success. However, it seems difficult, for some techniques at least, to head to the workbench for an initial test, regardless of how complete the description is. Implementing a technique is not an "intellectual" task, but rather a technical task that can only be well learned in a practical training course. Our descriptions must enable you to choose which training course will be best adapted to the problem presented on the given material. Thanks to our shared experiences, we have listed the limits and imperfections of the techniques discussed for many types of materials. However, we do not claim to present all the variations and adaptations of techniques that may have been developed here or there with success.

Everyone knows the techniques most commonly used in their field, but do they know the ones used in other disciplines? Curiously, we realize that the process leading to the selection of the technique is the same in all disciplines: knowledge of one's material, the methods of action of the techniques considered, and the requirements of the mode of observation planned. We also realize that a technique considered classic in one discipline may be poorly known in other scientific areas. Ultramicrotomy is probably the best example of a technique that had been bringing joy to biologists for the past 50 years before materials researchers became aware of its strengths as well as its limitations. By knowing the actions coming into play in each type of technique, we invite you to think about what is going on during preparation. This will enable us to predict whether or not our material will be damaged by preparation. We thus train our critical minds by improving the recognition of artifacts and refining the interpretation of our results. Technique is just like cooking, but scientifically reasoned cooking has a much greater chance of being effective and reproducible.

Today there are still too few interdisciplinary bridges due to a lack of relationships, communication difficulties, and/or hyper-specialization. But these bridges are essential to resolve the problems of materials that grow more and more complex and often involve mixed and composite materials. This work is aimed at the latest generation of microscopists, the researchers in emerging disciplines who need to characterize their new materials, and industrial researchers who are often confronted with never-before-seen problems that are sometimes far removed from their base training. In this compilation, they will find the ideas that are indispensable to understanding their problems and the means for solving them. This work might also be of great service to those who make it their calling to be open to all, such as technical platforms and joint imaging and analysis centers.

Yes, this was an adventure that carried us through 5 years of work in spite of ourselves. From being highly professional, our meetings also became very friendly, with bitter and heated debates to be sure, but always in the spirit of serving science rather than some personal flattery. Oh, how many things we learned in the course of those 5 years! First, in the disciplines that we were not familiar with, in the strictness of expression striving for a more universal language, and last, in the art of using all of the resources of a computer, including those for maintaining long-distance relationships between the various partners. Many times we had to go back

to the drawing board, or to colleagues, to confirm an idea or illustrate a proposal. We would like to thank them wholeheartedly for their diligent and effective assistance. It was a lovely undertaking and a truly shared one, with each bringing their skills to the service of the common cause. It was a marvelous human adventure that will leave its mark on our professional relationships.

We would like to thank our various supervisors for agreeing to give us the time to do this and for the two retirees, thanks goes to their families for understanding the worthiness of this commitment. We also thank those who helped us technically speaking, including Michel Charles and the CNRS-Formation department in the creation of the web site preparation guide, Frédéric Lebiet for setting up the web site, Avigaël Perez for creating the diagrams, Bernard Lang for translating the web site sheets into English, Aurelien Supot and Michael Healey from Atenao Company for the translation of the French version of the books into English, and Joseph McKeown (Arizona State University, Tempe, USA) for the review of the final English manuscript.

Our gratitude most especially goes out to our colleagues of the LM2C laboratory of CNRS UMR 8126 at IGR and the CIME of EPFL of Lausanne, for their moral support, their help, and their precious advice on the creation of this collective work. We would like to thank those who supported us morally and financially in our undertaking: CNRS-Formation and the French Microscopy Society. Last, Gérard Lelièvre, Director of the MRCT of the CNRS, deserves special recognition. He supported us very early on in our approach and gave us the material means for this creation. We owe the publication of this book to him.

Villejuif, France — Jeanne Ayache
Paris, France — Luc Beaunier
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Jeanne Ayache CNRS researcher in materials science and biology, Molecular and Cellular Microscopy Laboratory, CNRS-UMR8126-IGR Mixed Research Unit, Institut Gustave Roussy, Villejuif, France.

Jeanne Ayache is a CNRS physicist and microscopist researcher. Since she joined the CNRS in 1977, her research activities have been focused on studying the structure of materials belonging to the interdisciplinary fields of materials and earth sciences. She especially studied the structure of natural and industrial carbon-based nanomaterials, superconducting ceramics, oxide-based thin film, and heterostructures, down to the atomic or molecular scale. She is now working in the life science research field, at the Cancer Institute Gustave Roussy UMR 8126 of CNRS in Villejuif, France, where she is developing the aspects of electron microscopy in cell biology.

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Luc Beaunier is a CNRS researcher in physics in the Electrochemical Interfaces and Systems Laboratory at the Université Pierre et Marie Curie, Paris, France. His research activities in the physical metallurgy fields are related to corrosion phenomena induced by chemical and physical defects in metals. His last research interest is surface-alloyed metals by light energy laser treatment. All these materials are characterized by electron microscopy and spectrometry analysis (TEM, SEM-FEG, EDS, PEELS).

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Jacqueline Boumendil was technical director of the Center for Applied Electronic Microscopy in Biology and Geology CMEABG at the Université Claude Bernard-Lyon, Villeurbanne, France, and is now retired. The 37 years she spent in this center led her to study many normal and pathological biological samples, as well as structure of new polymeric materials. She has set up training in electron microscopy

sample preparation techniques that she taught for over 20 years. She has been in charge of the development of these techniques and particularly the cryotechniques.

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Gabrielle Ehret was technical director in transmission electron microscopy at the Laboratoire de Minéralogie et Cristallographie, then at the Institute for Materials Physics and Chemistry, Strasbourg, France, and is now retired. Since she joined the CNRS in 1970, her specialty has been the study of minerals, catalytic samples, and nano-carbon specimens. She was in charge of the transmission electron microscope training and teaching for the new TEM users and student research support.

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Daniéle Laub is technical director of microscopy sample preparation at the Lausanne Federal Polytechnical School (EPFL), Lausanne, Switzerland. Since she joined the CIME (Centre Interdisciplinaire de Microscopie Electronique) in 1988, she has been in charge of the development of sample preparation techniques for different types of materials (polymer, metal, semiconductors, ceramics, catalyst, etc.). She is responsible for sample preparation techniques training and teaching to new TEM and SEM users.

Contents

Chapter 1
Techniques: General Introduction

The second volume of the "Sample Preparation Handbook for Transmission Electron Microscopy" contains descriptions of 14 preliminary and/or complementary sample preparation techniques and 21 thin slice preparation techniques for the transmission electron microscope (TEM).

The operating mode for each technique includes the principles behind the technique, the experimental conditions for performing it, and the operating modes as well as the possible preparation variants, advantages, limitations, and characteristic artifacts produced by the technique. In this second book, the reader can also find information about the current fields of application for each technique, the types of materials prepared as well as their conditions, the possible TEM analyses based on each preparation, and the possible hazards encountered during the preparation. Before using each technique, it is indispensable to read about it in the corresponding section in the Chapter 4 of first volume "Methodology".

Investigations (SEM, AFM, SIMS, etc.) of the material surfaces require flat and smooth surfaces, sometimes down to the atomic scale. Certain preparation techniques presented in this book can be either partially or wholly used for the preparation of samples in the fields of optical microscopy, scanning electron microscopy, atomic force microscopy, or even ionic microscopy.

The characterization of materials using the TEM requires the use of samples that are thin enough so that they are transparent to electrons (thicknesses between 20 and 100 nm). In order to do this, it is often necessary to make the samples small enough to fit in the electron microscope specimen holder, i.e., 2.3 or 3 mm in diameter, depending on the type of microscope or the analysis to be made. Then, their thickness must be reduced while keeping the final thinning technique in mind. This step falls under the domain of *preliminary preparation*. For unstable and hydrated biological materials, preliminary preparations include fixation and embedding steps. The next step, which results in the thin slice, constitutes the *preparation technique*.

Lastly, if the sample cannot be observed in its current state after the last step (e.g., because of poor contrast), one or more complementary techniques are required. This additional preparation falls under the field of *complementary preparation*.

All of the techniques detailed in this book can be found in a more abridged form online at http://temsamprep.in2p3.fr . This web site mainly contains a methodological guide that allows the user to input some data on the properties of their material

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and the TEM analyses to be conducted, and to then select the possible technique(s) based on the information provided regarding the limitations, advantages, drawbacks, and artifacts for each technique.

On this site, the user also has access to a photo gallery of images containing the different types of samples as well as examples of artifacts produced during the preparation techniques for different types of materials. Every user can stock this database by adding a new preparation or TEM image complete with a description of the preparation technique used. The interest lies in offering the international community the widest possible variety of techniques and the most appropriate preparation depending on the type of material and the type of analysis to be carried out.

The sample preparation techniques are comprised of direct and indirect preparation methods.

Methods are said to be *direct methods* if they can lead to the preparation of a thin slice that is directly observable under the microscope in order to conduct a structural analysis, i.e., to use the TEM to determine the morphology, structure at different scales, crystallographic organization, chemical composition, and chemical bonds making up the material. The majority of techniques in materials sciences are direct ones.

In an *indirect method*, the sample itself is not analyzed, but rather an impression of the sample surface is made and analyzed. This is similar to the analysis of direct or indirect replicas. These techniques can only be used to characterize either surface topography or internal surface morphology (freeze fracture). The only indirect technique that enables the analysis of the surface and internal particles in a matrix is the extractive replica technique. In this case, structural and chemical characterization of particles can be performed.

In *Chapter 2*, among the preliminary preparation techniques, we will find the following:

Techniques specific to materials science: sawing, ultrasonic cutting, dimpling, sandwich, mechanical polishing, electrolytic polishing, and chemical polishing.

Techniques common to materials science and biology: embedding, substitution–infiltration–embedding at room temperature, substitution–infiltration–embedding in cryogenic mode, continuous support film, and holey support films.

Techniques specific to biology: chemical fixation and physical fixation: cryofixation.

In *Chapter 3*, among the thinning techniques specific to materials science, we will find the following:

Preparation techniques using chemical and electrolytic thinning: twin-jet and full-bath chemical thinning, and twin-jet and full-bath electrolytic thinning.

Preparation techniques using ionic thinning: ion milling thinning and focused ion beam (FIB) thinning.

In *Chapter 4*, among the purely mechanical preparation techniques, we will find the following:

Mechanical preparation techniques: crushing, cleaved wedges, tripod polishing (essentially used for materials science), ultramicrotomy, and cryo-ultramicrotomy (essentially used in biology and for polymer materials).

The majority of the preceding techniques are carried out after a mechanical thinning of the material or after a preliminary preparation.

These techniques, with the exception of the crushing method, help maintain the microstructure and orientation of the material as in its original form.

In *Chapter 5*, we will find the replica techniques common to materials science and biology. Among them we find direct replica, indirect replica, extractive replica, and freeze fracture.

Direct or indirect replicas are used for materials that cannot be damaged (collection materials, etc.), for irradiated materials which cannot be inserted into the TEM or for materials that are too difficult to prepare using the other techniques.

Extractive replica is essentially used in materials science to analyze surface particles or those extracted from a matrix.

Freeze fracture is used in biology to analyze the topography of internal surfaces in hydrated materials.

In *Chapter 6*, we will find the techniques specific to fine particle materials, which correspond to direct methods and concern both materials science and biology.

The dispersion of fine particle materials techniques are used in materials science and biology for materials composed of isolated small particles.

The frozen hydrated film of single-particle technique (or plunge freezing) is a technique specific to biological materials or polymers in suspension, in particular to single macromolecular particles.

These techniques result in random orientations of the particles.

In *Chapter 7*, we will find the specific contrast enhancement and labeling techniques. They are specific to biological materials and, generally speaking, to organic materials (polymers). Among them we find the following:

The decoration-shadowing technique, which is used in materials science and biology.

The "negative-staining" and *"positive-staining" contrast techniques,* which are principally used in biology, but also for polymer materials and nanomaterials.

The immunolabeling technique, specific to biological materials, which localizes functional proteins using labeling.

Among all of these techniques, we will also find the *cryo-methods* which constitute a group of preliminary preparation and thinning techniques involving the freezing of the material. They principally concern the investigation of biological materials and polymers and include the following:

– Physical fixation or cryo-fixation
– Substitution–infiltration–embedding in cryogenic mode
– Cryo-substitution
– Cryo-ultramicrotomy
– Freeze fracture

Conclusion

The nature and properties of the material to be characterized, along with the specific material problem, will determine our choice and lead to the use of one technique over another. The best technique will be the one that enables the determination of the intrinsic microstructure of the material without adding extrinsic defects. *Initially, it will be important to verify the validity of the technique chosen* by comparing the microstructure results obtained from at least two different methods and to not be satisfied with just one method.

Chapter 2
Preliminary Preparation Techniques

1 Sawing

1.1 Principle

This technique is used to make slices of bulk samples in order to reduce their dimensions and then prepare them using other preparation or finishing techniques (final thinning for transmission electron microscopy). In most cases, this means obtaining a slice with parallel faces of the right thickness (a foil or disk 1- to 0.1-mm thick).

The sawing technique uses the mechanical effect of abrasion. The friction of the material grains (abrasive), which are harder than the specimen to be machined, cuts precisely into materials. A fluid must be used, serving as both lubricant and coolant.

This technique is used to cut practically all materials (bulk and multilayer, compact or porous) of any shape. They can be single phase or multiphase, from very hard to soft. Fine particles must be embedded so as to make them into a machinable block.

1.2 Operating Mode

1.2.1 Equipment and Supplies

There are two types of wheel saw and one type of wire saw.

Wheel saws are driven by an adjustable-speed electric motor turning an axle on which a cutting wheel or saw blade (toothless) containing abrasive grains is mounted. To prevent flutter during rotation, the wheel or blade is held in place by rigid, stabilization flanges (reinforcement flanges) that have a smaller diameter than the wheel or blade. The saw uses friction to cut the sample and wear it down. A lubricant is used to facilitate the cut, prevent mechanical blockage, and keep the frictional temperature from getting too high.

The difficulty lies in selecting the wheel/abrasive/lubricant combination with regard to the material's properties, and then in selecting the speed/cutting force combination. Manufacturers offer pre-made solutions, whose purchase price must

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be taken into account. The blade contains the abrasive, and the binder, which may be made of metal or synthetic material, constitutes the blade's matrix. The binder is an important factor in cutting because if the abrasive grains wear or break down during cutting, they must be replaced by the underlying grains embedded in the binder's bulk. This cannot occur unless the binder also wears away. The binder/abrasive combination is determined by the manufacturer for a given application or material. Therefore, the manufacturer's instructions must be followed. Wheels with a diameter ranging between 50 and 300 mm are available commercially.

A small blade wheel is more stable than a large one and provides greater cutting precision. The thicker the blade, the more stable the wheel, and therefore, the better the precision. However, the rate of material removal decreases, and therefore cutting speed decreases as well. Cutting wheels are generally made with a metal binder (bronze, nickel alloy, etc.) or a so-called resinoid binder (bakelite, etc.) with the abrasive embedded in the bulk. The wheel thickness increases as a function of an increase in wheel diameter in order to have increased stiffness:

- Metal: Thickness between 0.2 and 2 mm for diameters between 50 and 300 mm.
- Resin: Thickness between 0.3 and 1.6 mm for diameters between 100 and 230 mm.

Metal blades contain abrasive grains in their bulk or in a coating (made using galvanization or chemical or electrochemical deposit), while the abrasive is always embedded in the bulk for resin wheels. The composition, dimension, shape, and quantity of the abrasive grains are crucial (see the chapter on mechanical polishing). The most commonly used abrasives are silicon carbide, diamond, alumina (corundum), and boron nitride. There are also wheels made entirely from abrasive grains (alumina, silicon carbide); these wheels are compressed together without a binder, but are thick enough to maintain their rigidity and solidity. They are very effective on all materials if a high loss of matter is not important.

Examples of uses are as follows:

Tool	Use	Application
Metal binder, diamond	Rough cutting	Carbide, steel, composite
Special metal binder, diamond	Rough cutting	Refractory, glass
Metal binder, boron nitride	Rough cutting	Very hard material, steel
Resin binder, diamond	Fine cutting	Sintered carbide, ceramic, mineral, glass

During cutting, the abrasive is used to remove shavings, shards, or splinters of the sample. The abrasive can wear out or crack, meaning that after a certain period of time, the blade is no longer effective for wheels where the abrasive is only embedded on the surface. For wheels containing abrasive in the bulk, the binder wears away, exposing new grains and allowing the wheel to be used for a longer period of time. A wheel using a soft binder such as bakelite (resin) minimizes the roughness and

cutting deformations. This is because the resin wears away very quickly, exposing the new abrasive grains, which are at maximum effectiveness. The wheel wears out quickly, but it is not expensive to replace. Wheels with metal binders wear out slowly and tend to become clogged, but they can be used for a long period of time.

The size and shape of the abrasive grains influence cutting effectiveness as well as the damage caused to the sample. The force penetrating the sample must also be taken into consideration in addition to the cutting speed, albeit to a lesser extent. As more pressure is applied, more mechanical damage is induced and the more the material is heated. The speed of the blade not only determines the penetration in the sample but also induces heat.

The lubricant is an important factor. Lubricants are generally water, alcohol, watchmakers' oil, or glycerin, but there are also solutions specific to each manufacturer. In all cases, it is critical to use a lubricant that serves as a coolant in order to prevent the temperature of the cutting wheel and the specimen from rising. The lubricant also helps to clear away cutting waste. The lower portion of the saw blade either dips into a tray containing the lubricant or a nozzle supplies the cutting zone with the liquid, which is filtered, cooled, and then reused.

The sample is firmly attached to a specimen holder. If the sample is fairly bulky, a mechanical blocking system that consists of screws and clamps is used. If the specimen is small, a hot or cold adhesive is applied by using hard glues or waxes on a glass or metal plate that will be attached to the specimen holder. In order to make oriented cuts, a specimen holder equipped with a goniometer head is used. This can be moved to an X-ray diffractometer in order to verify the orientation.

A. *In the first configuration*, the saw is a metal wheel and the specimen advances by means of gravity. This is the most widely used configuration. The blade is metallic (bronze, nickel alloy, etc.) and relatively thick, and in this case, the abrasive grains are located only on a peripheral exterior strip that is typically 1-cm thick (produced using galvanization, chemical or electrochemical deposition, or mechanically). They are often contained in the metal's bulk or in a coating. This type of wheel is generally used for ductile or resistant materials. This wheel has a certain thickness (a few tenths of a millimeter), but has the advantage of remaining rigid. It is generally used at a slow rotational speed (from 1 to 300 rpm).

The specimen is placed on the specimen holder at the end of an arm (Fig. 2.1). A counterbalance system (front and back) is used to adjust the penetrating force depending on the material type. In this case, the specimen rubs on the saw blade by gravity. Blade advancement is therefore controlled by the pressure caused by gravity, the rotational speed of the saw blade, and by the effectiveness of penetration, which depends on the material and the choice of abrasive. During cutting, the blade's cutting edge can be honed by rubbing it on an abrasive block (alumina, etc.) that will remove any material and lubricant deposits.

In this configuration, there is a compressive stress applied between the sample and the blade. This causes a rise in temperature and mechanical damage. The difficulty lies in finding the right weight so as to prevent significant damage. Blade advancement is evaluated by sight, and it stops automatically once there is no longer

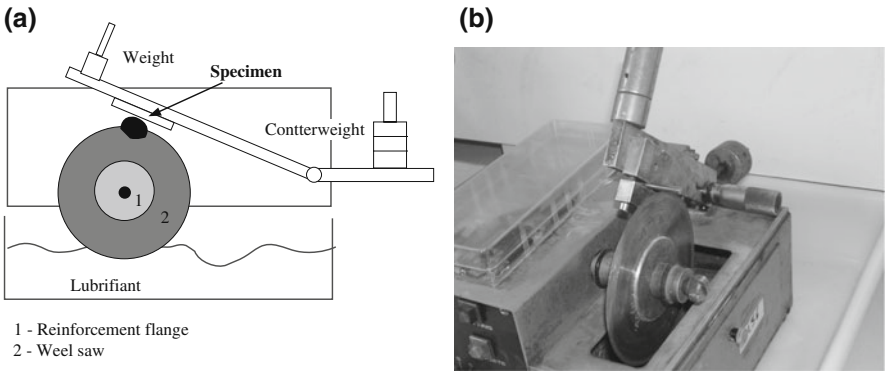

Fig. 2.1 **a** Wheel saw (diagram). **b** Wheel saw

any cutting effort. It is possible to cut slices down to 100-μm thick. For example, the cutting time for a 3-mm-diameter nickel bar is 7 min.

B. *The second configuration*, which is less widely used but more effective, is the sawing machine with a hydraulic platform. The specimen is put on a specimen holder that is placed on a vertical pillar, which is then mounted on the platform (Fig. 2.2). The advancement speed of the sample toward the saw blade is controlled; it is moved either using hydraulics or directly by means of an electric motor. The wheel is made of resin and is referred to as resinoid (bakelite). The rotational speed for this type of wheel is between 0 and 3,000 rpm. In this case, the abrasive grains are distributed evenly throughout the entire bulk of the wheel and it is generally used for hard and brittle materials. These wheels are thin and slightly stiff. In order to make them stable and effective, they are used at high speeds.

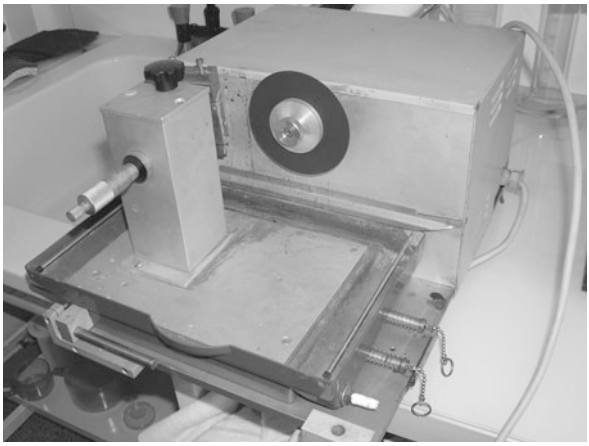

Fig. 2.2 Hydraulic platform saw

Hydraulic platform saw: The platform's movement is controlled by a piston (mounted underneath the platform) filled with pressurized oil that is evacuated through an orifice with an adjustable opening. This controls the speed at which the platform moves toward the specimen. In practice, the platform is pulled backward to fill the piston with oil. Pressure on the oil is maintained using a spring. The adjustable micro-escape valve controls the oil outflow and therefore the speed of the platform. The fluid circulates in a sealed closed circuit. Advancement and penetrating force (which must be minimized) are regulated by the hydraulic advancement of the platform (0–3 mm/s).

Motorized platform: The platform is moved by an electric motor that is programmable from 0.005 to 3 mm/s.

In both cases, the advancement speed must obviously not be faster than the penetration of the saw blade into the material; otherwise, there is a risk of breaking the blade and/or destroying the specimen. The advantage is that the sample is not subjected to any stress other than that created by the formation of shavings under the effect of abrasive grains (there is no effect from the weight of the saw, as with a simple wheel saw). Thus, brittle specimens are handled more easily and mechanical stresses are minimized. In this configuration, a micrometric sensor constantly measures the cutting progress. There is no temperature rise, no structural change, and no deformation. This saw uses resinoid disks that rotate at high speeds. Another advantage is that because all of the movements of this configuration are controlled, the thickness of 3-mm-diameter slices can be corrected while cutting rather than having to do this by polishing.

In both configurations, a micrometric screw is used to precisely adjust the specimen's position with regard to the saw blade's position, e.g., to make successive slices.

Wire saw (Fig. 2.3): This technique is even gentler than the abrasive resinoid-disk technique and is used to cut very brittle samples with very high precision. The specimen is screwed onto a fixed specimen holder or stuck to a support, e.g., by using thermal glue. The cutting wire is a bronze or stainless steel wire that is approximately 10 m in length, with a diameter as small as 30 μm. Therefore, all of these operations must obviously be performed with care. The wire is stretched either horizontally or vertically between two rollers, enabling endless or reciprocal movement, thus cutting the sample. The speed of the wire is variable up to 4 m/s. The wire is stretched like a bowstring and lies on the specimen with no application force. The wire is loaded with abrasives, generally with diamond granules 5–60 μm in diameter, inside the bulk or deposited on the surface by galvanization or chemical or electrochemical deposit. It is cooled by a tube supplying the coolant liquid (water or lubricant) or it bears a lubricant containing loose abrasive freely. In the configuration using a horizontal wire, a counterweight system regulates the penetration force of the wire (gravitation). In the vertical wire configuration, mechanical advancement is controlled by an electric motor. In both cases, the cutting progress is constantly measured with a micrometer. Cutting can be monitored using a stereoscopic microscope placed in front of the sample. Cutting can be very slow (2 mm/h) but can reach 3 mm in a few minutes on a metal bar. With this sawing system, there

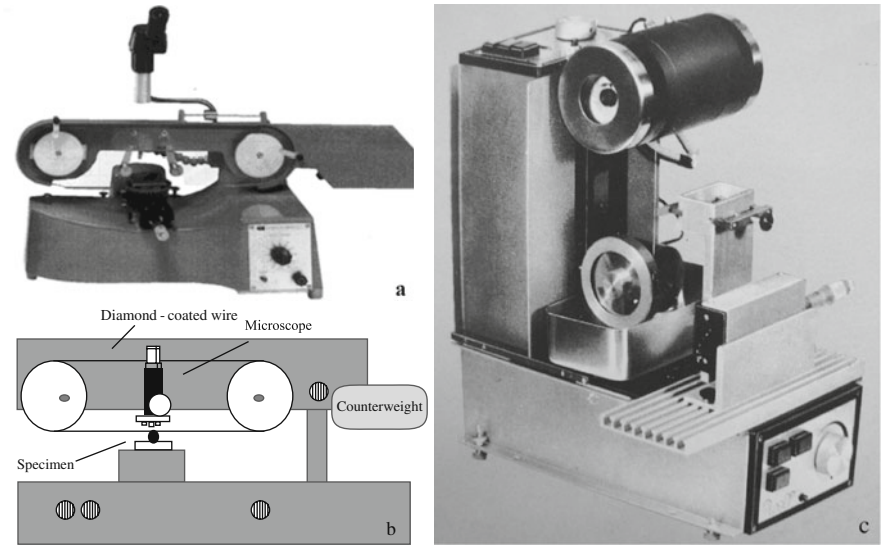

Fig. 2.3 Horizontal (**a**, **b**) or vertical (**c**) wire saws

is no rise in temperature, no structural modification, and no deformation. The cut is smooth and scratch-free.

1.2.2 Procedure

The parameters involved in cutting are as follows:

1. The nature of the sample, depending on whether the bonds are metallic, semi-metallic, covalent, or ionic, as well as the hardness, ductility, and brittleness of the sample.
2. The nature of the abrasive: material and performance of grains, the nature of the friction.
3. The lubricant: helps with abrasion, either direct (corrosive) or induced (reactivity of the cut bonds) chemical reactivity.
4. The technique: metallic or resin wheel saw, wire saw.
5. The load or pressure exerted.
6. The abrasion speed.
7. The cutting time

The choice of equipment is crucial to protect the sample from mechanical damage, depending on the objective: a shaping cut or a cut to make a thin slice with parallel faces for polishing or final thinning. In the first case, a rather rough cut

is acceptable, but in the second case, it is always necessary to work with extreme gentleness. The final objective guides the user toward a certain type of saw, blade, and lubricant.

These techniques use a mechanical effect as follows:

- Two bodies in the case of the saw: The cutting wheel consisting of the abrasive set in the binder rubs on the sample (cutting wheel and sample)
- Three bodies in the case of the wire: The wire drags a loose abrasive against the sample (wire, abrasive, and sample)

The friction of the abrasive grains creates wear, causing the blade or wire to penetrate the material. The abrasive concentration plays a major role, as does the addition of a lubricant.

Depending on the material's properties, the sawing machine and cutting blade (binder and abrasive) to be used are generally chosen following the manufacturers' guidelines. The use of a lubricant is very important for minimizing mechanical damage. It also serves to clear away waste from wear and to minimize the thermal effect. The lubricant is usually water, but manufacturers offer cutting products containing oil, glycerin, and other undisclosed compositions.

The sample is mounted onto the specimen holder. The weight for applying the penetrating force is determined. A heavy weight on the sample or a rapid cutting advancement for platform saws works quickly, but at the cost of a relatively violent mechanical effect on the object. A lighter weight or slower advancement prolongs the cutting time, but minimizes the pressure. Depending on the type of wheel, the cutting speed and quantity of the lubricant are then chosen.

The parameter that needs to be controlled in order to prevent mechanical damage is the rotational speed (or scrolling speed for the wire saw): the wheel material determines the speed. For a metal blade, a moderate speed is used to prevent temperature rise and give the abrasive time to erode the material. For resin wheels, it is necessary to work at high speeds to maintain blade stability through centrifugal force. In all cases, the cut must be made as quickly as possible in order to reduce the interaction time with the material and, consequently, the creation of defects.

1.3 Variants

1.3.1 Annular Wheel Saw

This relatively rare technique is similar to wheel sawing; however, in this case, the blade is in the form of a ring, held by its outer perimeter (hooping). The inner edge of the ring cuts the sample. This configuration provides great blade stability and therefore minimal flutter, resulting in a very high precision cut (Fig. 2.4).

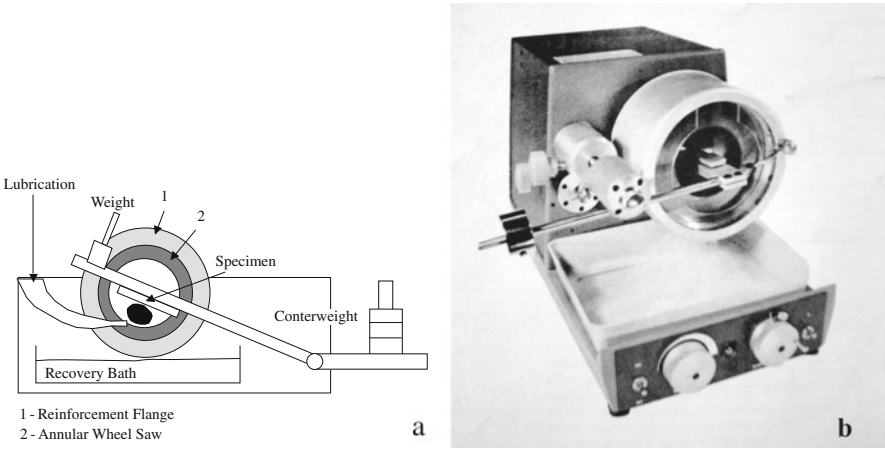

Fig. 2.4 **a** and **b** Annular wheel saw

1.3.2 Electrochemical Cutting

Electrochemical saws, based somewhat on the "wire cheese cutter" principle, are used to cut metals (conductors). They act using anodic electrochemical machining. An electrolyte jet is forced through a tube. A wire centered in the tube is stretched out and serves as a cathode. The sample is the anode. Therefore, there is a solid wire surrounded by a cylindrical electrolyte film. The common axis of the wire and tube is either vertical or horizontal, depending on the equipment design. The assembly is motorized in order to advance it toward the sample. An electrical power generator provides high current densities for rapid dissolution using an anodic electrochemical process. Highly conductive concentrated salt-based solutions that can support high current densities are generally used for electrolytes. The wire's advancement is motorized. This method is used to make cuts without physical contact with the sample, producing a gentle cut that does not introduce any defects into the sample. This technique is perfect for cutting single crystals according to the crystallographic planes when the sample is oriented crystallographically on a goniometer head. Using this method, 100-μm-thick slices of 3-mm-thick bars can be made. The cutting line is around 0.5 mm.

The benefit is that there is no force applied to the sample. The cut has the same surface quality as in electrochemical polishing and causes no mechanical damage. This type of saw is extremely rare in laboratories and is found more frequently in industries. It is used only on metals.

1.3.3 Acid Saw

This technique is the same as the wire saw, but there is no abrasive used. The wire directly carries a strong acid that acts to chemically dissolve the sample.

1.3.4 Spark Erosion Cutting

An electrical generator supplies electrical pulses, creating a series of successive sparks (like short circuits) between the tool (which is the electrode) and the sample for slicing, drilling, or trepanning without the mechanical damage associated with conventional machining. Both the cutting tool (a horizontal or vertical wire or metal blade) and the sample are placed in a dielectric liquid bath (e.g., oil). The tool's lowering movement is motorized. The specimen wears, causing a cutting line, which produces damage that can reach a certain thickness (0.1–0.8 mm). There are two types of procedure: either a wire spark or blade spark erosion cutting for simple cuts (like a saw line) or by a punch, where the tool has the desired shape to make any sample, depending on the shape of the electrode (round, square, triangular, etc.). In this way, we can produce 3-mm disks for use in electron microscopy. It is not a widespread method, but it is very effective (Fig. 2.5).

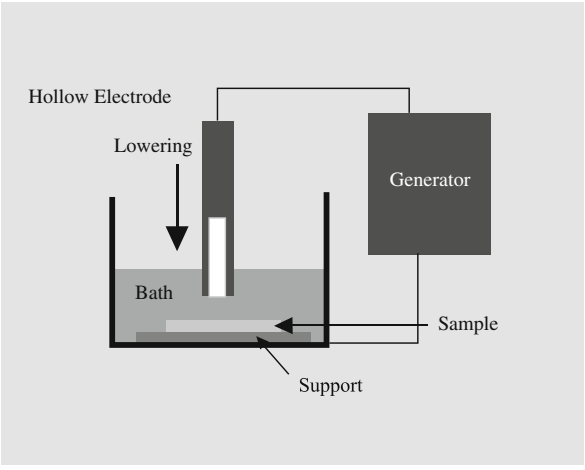

Fig. 2.5 Spark machining unit

1.4 Advantages

These sawing techniques are used to make cuts in bulk specimens. They are mainly used for specimen and plane surface cuts prior to other initial preparation or finishing techniques. These cuts yield thin parallel-sided sections, down to 100 μm and sometimes thinner. Mechanical damage is minimized (2–10 μm in depth) compared to saws with teeth (up to 1 mm in depth), regardless of material hardness.

1.5 Limitations

Despite all precautions, there is still mechanical damage, matter transport (except for the electrochemical saw), and pollution that must be cleared away. Because of

the mechanical effect, there is significant strain hardening for hard and brittle materials and deformation damage for ductile materials. If the abrasive chosen is not suitable or if the pressure is too high, cracks can be induced. This results in the loosening of substrate particles, thus damaging slice quality. There is also a rise in the temperature due to friction and abrasive grains can be left behind, embedded in the material.

1.6 Compatible Techniques

These cutting techniques are used before the following techniques: grinding, mechanical polishing, tripod polishing, dimpling, twin-jet electrolytic polishing, full-bath electrolytic polishing, chemical polishing, twin-jet chemical polishing, full-bath chemical polishing, sandwich technique, ion thinning, and FIB.

1.7 Risks

Risk of cut fingers on rotating saw blades.

1.8 Conclusion

These techniques are frequently used for the preliminary preparation of samples in materials science.

2 Ultrasonic Cutting

2.1 Principle

This technique is used to cut off a specimen using ultrasonic trepans of varying shapes on raw samples or samples that were previously reduced to small dimensions using sawing and/or polishing. These samples can then be worked on using other preparation techniques.

This technique uses an ultrasonic generator to vibrate a metal shaft. At the end of the shaft, a hollow cutting tool is vibrated laterally at a very low amplitude using ultrasound. A lubricant containing abrasive is placed between the tool and the specimen. The friction of the abrasive grains, which are harder than the specimen, cuts into the material with precision.

This technique is used to cut bulk, thin film, single-phase, or multiphase materials. It is not suitable for ductile, very brittle, or soft materials.

2.2 Operating Mode

2.2.1 Equipment and Supplies

A generator (oscillating crystal, e.g., zirconate/titanate) transmits ultrasound on the order of 26 kHz to a vertical metal tool. This tool is then subjected to very low amplitude vibrations. At its free end, it carries a toothless hollow cutting tool (plane section) that is subjected to these lateral vibrations (Fig. 2.6). This tool is either circular in order to produce a disk (or cylinder) that is 3 mm in diameter or rectangular (a few millimeters on each side) in order to produce a strip. The specimen holder is placed on a bulky, heavy magnetic metal support (either cylindrical or rectangular) that is wider than taller in order to ensure stability and positioning. The support is placed on the machine platform under a tool that is then lowered until it touches the sample. A lubricant containing abrasive (carbide, nitride, or boride) is placed between the tool and the specimen. A mechanical sensor constantly measures the progress of the tool into the material. This device is used to cut brittle materials such as ceramics, semiconductors, and minerals.

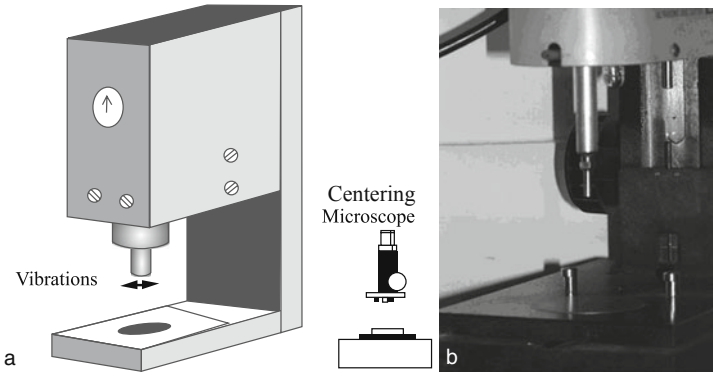

Fig. 2.6 **a** Ultrasonic grinder diagram. **b** Ultrasonic grinder

2.2.2 Procedure

In most cases, cutting is performed on a thin section approximately 100-μm thick (or less), but which can be as thick as 6 mm. This is used for sections produced by wheel or wire sawing or directly on wafers for ceramics and semiconductor materials. The section is hot glued to the bulk support to enable easy positioning on the machine platform. The support/sample assembly is placed under the tool to target the area to be cut out. A stereoscopic microscope may be attached for observation. A lubricant containing abrasive is placed between the tool and the specimen (this is three-body abrasion). The friction of the material grains, which are harder than the specimen,

cuts into the material with precision. A hollow cylindrical bit with an inside diameter of 3 mm is used to cut out a thin disk or cylinder from hard materials (the cylinder is then cut into cross sections using a wheel or abrasive wire saw to make thinner sections).

A rectangular tool is used to produce rectangular strips. These strips are used to prepare sandwiches: a sandwich is a stack of rectangular sections generally trimmed into wafers. The strips are then hot glued together under a press. In the cross-sectional direction of the stack, a cylinder is cut out using ultrasonic cutting in order to prepare a cross-sectional cut. It is essential to use a lubricant in order to minimize mechanical damage; this also serves to clear away waste produced by abrasion. The parameters that must be controlled in order to prevent mechanical damage are penetration speed, penetration force, and the quantity and type of abrasive and lubricant. A 6-mm-thick section is produced in a few minutes.

2.3 Variants

2.3.1 Hollow Bit

Ductile materials can be cut using a toothed hollow bit. For relatively hard materials, a motor-driven, rotating toothless bit (with an abrasive) can be used. The bit, with a 3-mm inside bore is mounted on a mechanical drill that is lowered manually or by a motor. This method is rarely used (Fig. 2.7).

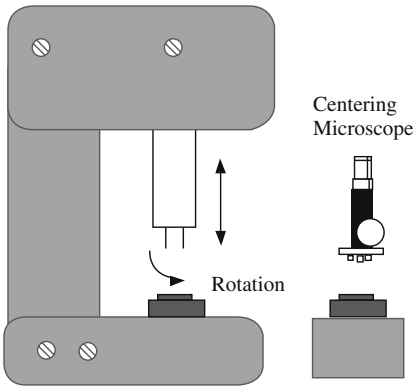

Fig. 2.7 Rotating grinder with a hollow bit

2.3.2 Punching (Stamping)

Metallic materials in the form of foils (100 μm) can be punched out into disk shapes if they are not too brittle. To prevent excessive damage to the entire sample, the foil is placed between 2 shock absorbers (paper, plastic sheeting, or metal foil). A piston presses on an anvil that has the required diameter (generally a 3-mm disk). It all

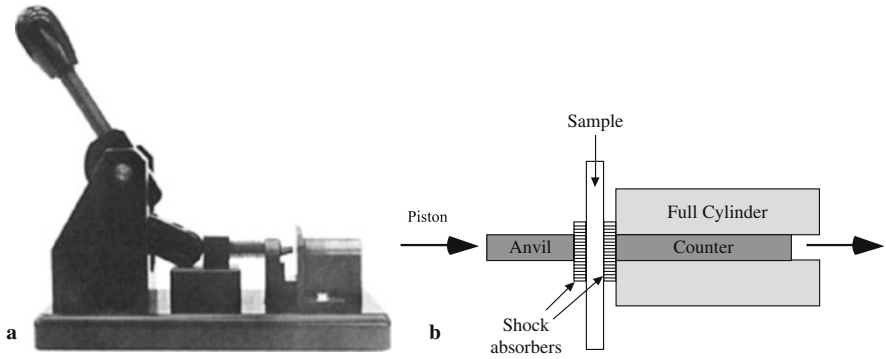

Fig. 2.8 **a** Punch. **b** Principle diagram

slides in a cylinder acting as a guide. This anvil is a plane section or in a slightly angled shape in order to progressively penetrate the sample (Fig. 2.8).

Behind the sample, a flat-section, spring-loaded counter-anvil holds the sample against the anvil, preventing significant deformation. When the piston is released, the counter-anvil returns the cutout disk to its original position and it falls into a tray. Mechanical damage is limited to a very narrow strip around the perimeter of the disk (500 μm), which does not reach the useful area in the center of the disk. This method is frequently used.

2.3.3 Chemical Cutting

Chemical cutting can be used for chemically sensitive materials such as semiconductors, minerals, and metals. To do this, a fairly thin slice (e.g., 0.1 mm) with a fairly large surface area of these materials is made using suitable techniques. Next, the slice is either hot or cold glued to a flat surface (glass, ceramic, or other). Self-adhesive strips in the desired shape (e.g., 3 mm) are then stuck to the upper side of the sample and it is all placed in a container. A chemical etching solution is added. The process is monitored using a stereoscopic microscope. After the unprotected surfaces are dissolved, the protective disks must be removed and the samples unstuck. This technique can be used to prepare several samples at once (Fig. 2.9).

2.3.4 Electrochemical Cutting

Electrochemical cutting can be used for a material that can be dissolved using an anodic process, e.g., semiconductors and metals.

To do this, a fairly thin slice of these materials (e.g., 0.1 mm) is made using suitable techniques. Self-adhesive strips in the desired shape (e.g., 3 mm) are then stuck to both sides of the sample and it is all placed in a container, while the slice is held with a conductive clip connected to the anodic pole of an electrical generator. A stainless steel plate connected to the cathodic pole is placed against the sample. The

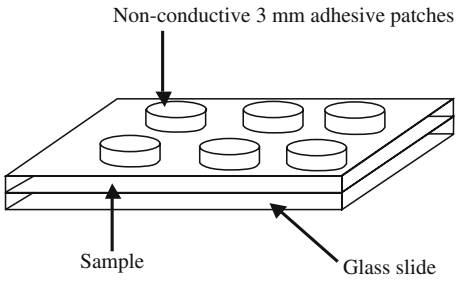

Fig. 2.9 Chemical cutting

diagram is the same as in Fig. 2.9. An electrochemical solution selected specifically for the material to be cut (see Chapter 2 Section 5 on electrochemical dissolution) is added. The operator monitors the process using a stereoscopic microscope. After the unprotected surfaces are dissolved, the protective disks must be removed and the sample unstuck. This technique can be used to prepare several samples at once. Another method consists of plating the sample and a conductive plate of the same diameter connected to the more anodic pole between two 3-mm cylinders of insulating matter in the container holding the cathode and the solution, and then dissolving the edges (Fig. 2.10).

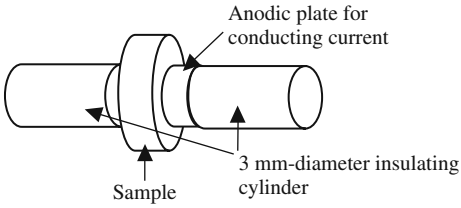

Fig. 2.10 Electrochemical cutting of a metallic disk

2.3.5 Electro-erosion Cutting

Conductive materials can be cut (slicing, drilling, or trepanning) using electromachining (or spark machining). Both the cutting tool (a horizontal or vertical wire or metal blade) and the sample are placed in a dielectric liquid bath (e.g., mineral oil, water) that reduces the temperature in the area being machined, removes residual metal particles, and enables spark creation. The distance between the tool and the sample is very small, from a few microns to 1 mm. The tool's movement is motorized. An electrical generator supplies the electrical pulses. A series of successive sparks (like short circuits) are created between the tool, which is the electrode, and the sample. The specimen is worn by fusion (8,273–12,273 K), producing the cut. The technique causes damage to the material that can reach a depth between a few microns and several hundred microns, depending on the device, but the damage is still less than with conventional machining. The process involves punching,

where the tool has the desired shape depending on the shape of the electrode (round, square, triangular, etc.), and thereby producing 3-mm disks for electron microscopy (Fig. 2.11).

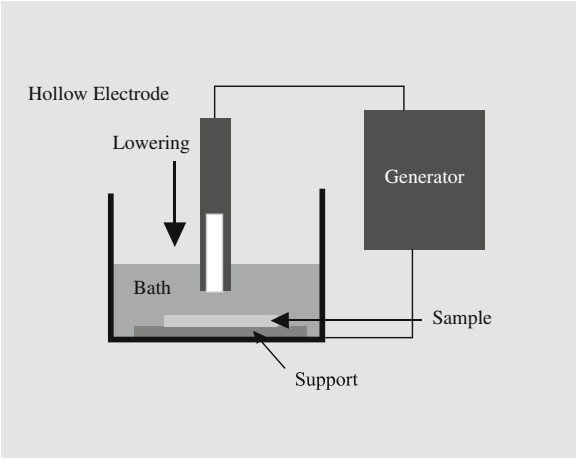

Fig. 2.11 Spark machining unit

2.4 Advantages

The advantage of this technique is that it works like a punch, i.e., it produces a sample with the desired shape and size in a single operation. Mechanical damage is minor and is generally located on the outside edges of the sample.

2.5 Limitations

Ultrasonic cutting cannot be used for the following types of materials: ductile, very brittle, soft, very soft, or very hard materials. Given the mechanical action, there is strain hardening for brittle materials and a thermal effect due to friction. However, the lateral extent is limited and does not reach the area that will subsequently be observed in transmission electron microscopy. The use of a lubricant is essential for minimizing these effects.

2.6 Compatible Techniques

This cutting technique can be used before the following techniques: mechanical polishing, dimpling, electrolytic polishing, twin-jet electrolytic polishing, full-bath

electrolytic polishing, chemical polishing, twin-jet chemical polishing, full-bath chemical polishing, sandwich technique, ion thinning, and FIB.

2.7 Risks

The use of ultrasound generates auditory risks. When preparing noxious materials (e.g., GaAs), there are risks related to aerosols.

2.8 Conclusion

This technique is certainly a more elegant and faster technique prior to preparation for ion, chemical, or electrochemical thinning.

3 Mechanical Polishing

3.1 Principle

This technique is used to produce smooth surfaces and thin specimens that have generally been obtained by sawing or ultrasonic cutting. This technique is also commonly used to subsequently prepare samples using other preliminary preparation or thinning techniques. In most cases, mechanical polishing is used to obtain a flat (parallel-sided slices), scratch-free surface, while reducing mechanical damage.

This polishing technique employs mechanical abrasion. The friction of the abrasive particles, which are harder than the specimen to be polished, removes material with precision. A fluid must be used; it serves as a lubricant and coolant and removes excess material particles.

This technique can be used to polish almost all bulk, compact, and thin film materials, and even fine particles or porous materials if they are embedded. The materials can be multiphase materials and can contain precipitates or segregations. The materials can be of any hardness, with the exception of very soft materials.

3.2 Operating Mode

3.2.1 Equipment and Supplies

The parameters involved in polishing are as follows:

1. The sample characteristics, depending on whether its bonds are metallic, semi-metallic, covalent, ionic, etc., and its hardness, ductility, and brittleness characteristics.

2. The nature of the abrasive: nature and performance of grains. The nature of the friction.
3. The lubricant: helps with abrasion, direct (corrosive) or induced (reactivity of bonds cut) chemical reactivity.
4. The applied technique: bonded abrasive (new polymer binders) or free abrasive.
5. The load or pressure exerted.
6. The abrasion rate.
7. Polishing time.

Commercially available rotary polishers, belt polishers, and fixed band or fixed platform polishers for manual polishing are used. Abrasive polishing is also used in dimpling. These preparations will be finalized using chemical, electrochemical, or ionic polishing.

Other polishing methods are used for special or very complex materials, as shown in the table.

Polishing technique	Principle	Application
Mechanical	Several polishing steps using an abrasive	Most materials
Mechanical/chemical	Addition of a reagent in the final mechanical polishing step	Refractory materials; alloys of W, Mo, Ti, Zr; minerals; and semiconductors
Chemical	Use of an etching reagent without an abrasive	Light metals and alloys; alloys with Pb, Cu, Zn, Al; minerals; and semiconductors
Electrochemical	Anodic dissolution	Metals and semiconductors, stainless Al, Ti-based alloys, noble metals
Electromechanical	Mechanical polishing with simultaneous anodic dissolution	Refractory metals

The initial sample is used as is, or embedded if necessary, and then cut. Several polishing stages must then be performed; these are determined based on the starting state of the sample and the desired objective. Depending on the case, the following steps are performed (from the coarsest to the finest):

– Rough grinding, lapping: initial corrective action to create a flat surface if one is not produced by cutting.
– Pre-polishing: polishing to flatten a rough surface after cutting or grinding.
– Polishing: fine stage to eliminate scratches and/or reduce sample thickness.
– Final polishing: step to obtain a shiny surface that must be completed by a final thinning.
– Super-finishing: obtaining a so-called mirror finish.

The objective is to achieve a flat surface by removing matter without inducing structural defects or chemical changes to the surface that cannot be subsequently removed. The result is a flat surface, as long as the surface is not dug into, creating deep internal defects. The surface is worn through successive stages so that the degree of roughness is reduced. Less matter is removed with each successive step because the abrasive grains are smaller and smaller. These are increasingly finer stages that require careful work and should be verified under an optical microscope, starting with the polishing step. In the end, the surface must not present any deformations or scratches.

The final objective may be a perfect surface, but for electron microscopy, the surface is always prepared with the aim of performing another technique; therefore, it is sufficient to end up with an acceptable roughness for finishing. In fact, some roughness is a definite advantage for chemical or electrochemical thinning.

The ratio of material hardness to abrasive hardness is an important parameter that must be considered at the start of the polishing process. Generally, at the finishing stage, an abrasive that is just one point harder than the material to be polished must be used in order to promote the simultaneous wear of the two bodies in contact.

Disk Polishers

Mechanical polishers are composed of one or more rotating metal platforms made of Dural, cast iron, nickel, or steel alloys (200–300 mm in diameter) that hold polishing platforms or disks containing an abrasive and a water source for cooling. The disk-shaped platform is rotated by a variable-speed electric motor. There are two types of polishers: routine high-speed polishers and those working at low speeds for finishing and super-finishing (Fig. 2.12).

Fig. 2.12 Rotary grinder/polisher

All manufacturers' catalogs provide user tables or charts to help guide the user in selecting supports, abrasives, and lubricants.

In the case of polishing platforms and disks, the abrasive grains are set into the support surface. This is called unguided erosion or two-body polishing: the abrasives

set into the support rub against the sample. The abrasive grains extend past the surface and act by tearing the sample's surface matter like a plow, somewhat roughly. The resulting surface is textured, contains parallel scratches and micro-shavings, and is rough.

The rough grinding, lapping, and pre-polishing wheels (platforms) are made of metal (e.g., cast iron, bronze, etc.) containing large abrasive grains in their bulk (diamond from 0.01 to 1 carat, carbide, alumina, etc.). They are used to grind very hard materials such as ceramics, tungsten carbide, refractory materials, and composites. The wheel is generally covered with a radial, circular, or crisscross pattern, in order to clear away particle debris. It can also have a grid surface containing the abrasive (diamond and silicon carbide). Rough polishing has a high removal rate; in all cases, it is rough and strong machining.

Pre-polishing (surfacing) or polishing disks are composed of a paper or plastic (polyester, etc.) support containing a fine abrasive (carbide, diamond, etc.) that is incorporated into the surface in a fixing binder and which is stuck to the support. The disk is either held in place by an external ring or is stuck on using an adhesive placed on the backside or is even held by magnets. This polishing has a low removal rate.

In all cases, a stream of lubricant (in most cases water, honing oil, glycerin, etc.) provides cooling and clears away particle debris.

For final polishing, cloths called pads or felt are used, on which a fine abrasive (diamond, alumina, etc.) is spread, either in the form of an abrasive powder with a lubricant (suspension and aerosol) or in the form of a paste. The abrasive grains are not fixed, but are held in place by the fibers (natural or synthetic). They have a gentler action than two-body polishing. This is called "three-body abrasion" (cloth-abrasive sample) and is referred to as "guided."

Polishing cloths can be divided into three groups:

1. *Woven*: These are made from different types of fibers. Their quality depends on the tightness of the weave. They have a rather hard action but prevent flared edges and relief of the specimen surface due to structural variations. Because of this, they are used for plane pre-polishing.
2. Unwoven (compressed): These contain various fibers mixed with or impregnated with plastic, elastomers, or latex. They are used especially for very hard materials, for mechanical/chemical handling of light alloys, and high-precision surface finishing (glass, quartz, sapphire, semiconductors, etc.).
3. Flocked: The fibers are implanted into the adhesive support. Their quality depends on the nature, length, density, and grade of the fibers. These are the only fibers to enable super-polishing (Figs. 2.13, 2.14, and 2.15).

Note that there are special cloths for alumina abrasive.

These three types of cloth can be divided into four classes:

– Rough, for abrasives from 45 to 9 μm
– Semi-finishing, from 9 to 3 μm
– Finishing, from 3 to 1 μm
– Super-finishing, from 1 to 0.1 μm

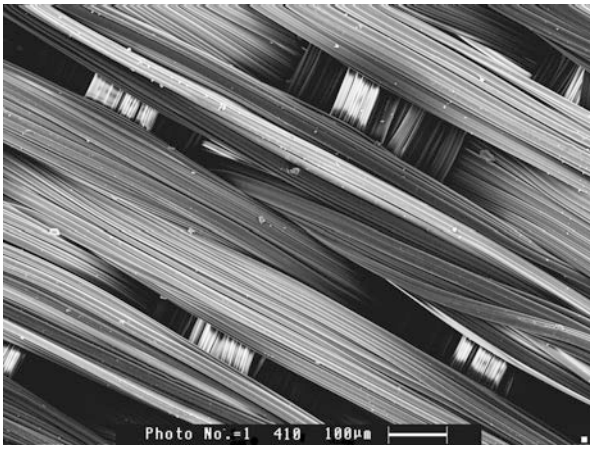

Fig. 2.13 Structure of a woven cloth

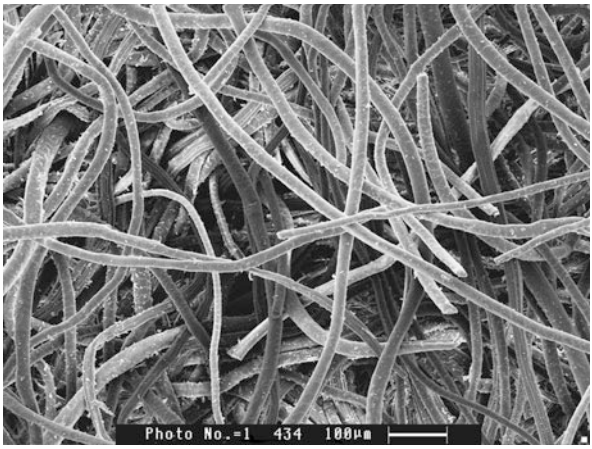

Fig. 2.14 Structure of an unwoven cloth

For flocked and unwoven cloths, the main parameter is fiber length. It is known that polishing will be more level on very short fibers where the abrasive grains rub on the sample, whereas on long fibers where the abrasive is coated, the sample floats on the fibers like on a carpet. This is why a woven cloth has a greater grinding power and provides better flatness. But for the same diamond grain size, it has a lower polishing quality than a flocked cloth because it acts more gently.

Another way is to place the loose abrasive powder between the sample and a bare rotating platform (generally glass) with a lubricant. This technique is used for the super-finishing of relatively hard and brittle samples like glass, minerals, geological samples, and electronic materials with diamond, alumina, reactive carbides, or oxides (colloidal silica, cerium oxide, etc.).

Fig. 2.15 Structure of a flocked cloth

There are also bulk or porous polyurethane-based binders containing grains of cerium oxide or zirconium, and graphitic Teflon polishers on glazed ceramic, Pyrex, or silica. Recent developments have brought about magneto-rheological finishing polishers, using magnetic and non-magnetic abrasive particles. The magnetic field is used to create chains and increase viscosity.

At any rate, is it still necessary to carry out super-finishing if the sample preparation is finished using chemical or electrochemical polishing? No, because these techniques are often more effective on surfaces that are slightly damaged mechanically. On the other hand, super-finishing is mandatory for tripod polishing.

Abrasive

For the abrasive, the chemical nature and structure must be taken into account, since they will determine its hardness and brittleness. There are many types of abrasives that will be chosen based on the nature of the material, the polishing support, and the finishing quality required. It is very important to know that an abrasive only acts through its hardness and the aggressiveness of its cutting edges, which govern its wearing power and the quantity of matter removed.

Abrasives are generally silicon carbides, chromium carbides, nitrides, borides, alumina, diamond, corundum, garnet, iron oxide, chromium oxide (for Si and metals), pure tin with lead or tin oxide (for soft metals and plastics), oxides mixed with cerium, lanthanum, and zirconium, diamond (for metals, semiconductors, and crystals), cerium oxide, zirconium oxide, and silica (colloidal), etc.

There are either natural (derived from natural minerals) or artificial (grown industrially) powder abrasives. Their shape and size vary widely. Either rough crystals with rounded shapes and therefore a moderate wearing action or ground crystals that have much more aggressive edges and work by cutting are used. This is the case

with diamond and alumina abrasives. For diamond, a distinction is made between monocrystalline and polycrystalline diamond in rough or fractured growth grains. There are also so-called super-abrasives whose matter and shapes give a much better yield for a markedly higher cost. These special abrasives are made of grains like interlaced carbide rods that are very effective but violent. The most commonly used abrasives on wheels with binders are silicon carbide and diamond.

Abrasives for cloths (woven, non-woven, and flocked) are available either loose in powder form or bound to a liquid lubricant, which can be either water soluble or not. There are some abrasives suspended in water, alcohol, or oil, which are packaged in spray guns, aerosols, or a paste to be diluted directly on the wheel. The aerosol spray is more useful for making a homogeneous distribution on the platform and proves fairly economical because less product is needed. The paste is more widely used because it is less expensive, although it is harder to use because the paste must be spread out homogeneously with a dilutant. In three-body polishing, the abrasive grains act by wearing as they roll on the sample. It is a gentle method used in finishing with finer grains. It must be performed with care as the abrasives are loose and therefore can become encrusted in the sample.

Diamond abrasives are used in the following ways:

- A high concentration of single-crystal diamond in water or alcohol for metals and steel
- A very high concentration of single-crystal diamond in water or alcohol for hard steel and ceramics
- A normal concentration of single-crystal diamond in water or alcohol for soft steel, light alloys, titanium, zircaloy, or minerals
- Normal concentration of polycrystalline diamond, in water or alcohol (exceptional cutting power) for the pre-polishing of hard steel, tungsten carbides, ceramics, and refractory materials

The size of the abrasive grains is graded. The table below provides the equivalence between the French standards (grade), US standards, and micrometric size.

French grade	US grade	Micrometers
100	100	162
120	120	125
180	180	82
240	240	58
320	310	46
400	320	35
600	380	26
800	400	22
1,000	600	18
1,200	800	15
2,400	1,200	10
4,000	2,400	5

The following are the most common loose powders:

- Silicon carbide from 40 to 7 μm: This is the most commonly used starting abrasive because of its efficiency at all grades and especially its cost. It will generally be followed by other abrasives.
- Diamond, 5–0.01 μm: Diamond presents exceptional hardness and resistance to crushing. Its cutting power is very good and it is even economical to use.
- Alumina, from 40 to 0.02 μm: Probably the most commonly used abrasive for finishing semiconductors, quartz, and electronic component materials. The grains become encrusted more so than the diamond grains.
- Boron carbide from 120 to 5 μm: An exceptional abrasive for all hard or brittle materials

For super-finishing of glass, silicon, and semiconductors, we use zirconium oxide, cerium oxide, colloidal silica, silica gel, chromium oxide, and magnesia.

Cerium oxide is a polishing agent for glass that combines the natural hardness of an oxide with a chemical reaction occurring at the interface of the cerium oxide and the silica. The result is a silicate layer that makes mechanical grinding easier.

Chromium carbide is used for very hard materials, but it is slower than diamond, tungsten carbide, ceramic, etc.

For maximum efficiency, the abrasive grains must either maintain their edges or renew them by fracturing or the binder must wear away to uncover new grains.

Sample damage is possible:

- Mechanical: inclusion or phase loss, cracks
- Thermal: heating, fusion
- Chemical: surface modification

As a rule, regardless of the abrasive grain size and material type, the specimen can be damaged up to a depth of three times the abrasive grain size. After gentle super-finishing, the depth affected mechanically can be estimated at one-third of the size of the abrasive grain.

Sample Holder

A large specimen (either embedded or not) can be held by hand for all of the polishing steps. Some know-how is required to maintain flatness and to prevent edge rounding. In most cases, a polishing guide is used. An embedded sample or a bar can be placed in a heavy and wide holding cylinder to ensure stability and flatness. This can be made for a minimal cost in the workshop. The sample must be either hot or cold glued to a support to make sure it is held in the polishing guide. A specimen holder is always used for finishing and super-finishing steps.

There are also commercially available specialized specimen holders. While fulfilling the same role as above, there is a scale to measure the thickness removed.

Some can also be used to correct a specimen's tilt. In automatic polishing machines, an arm holds the specimen holder, which ensures a planar or planetary rotational movement, provides pressure, and controls the advance of the wear (Fig. 2.16).

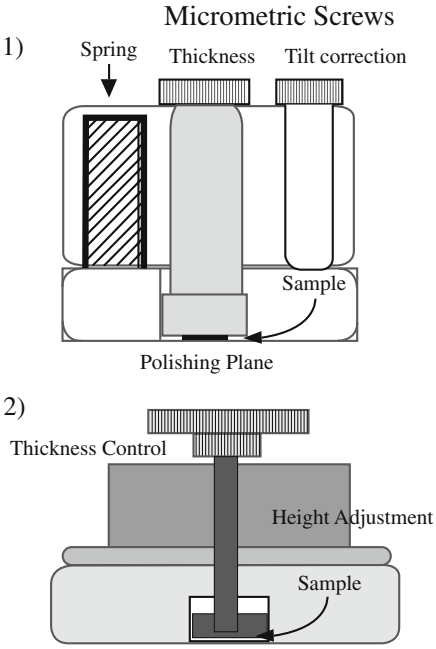

Fig. 2.16 Two manual specimen holders, one with tilt correction

If the pressure applied on the sample is too high, polishing will be ineffective and the surface will be destroyed. However, high pressure can, however, be used intermittently on the sample at the start of rough grinding and, paradoxically, during final polishing by successively lighter loads. A pass is made through the polisher, the debris is removed, or polishing is continued while changing the path. Ideally, no more than a third of the diameter of the diamond grain is penetrated.

Sample Cleaning

The sample must be cleaned at each step in order to prevent bringing abrasive particles from the previous step into the subsequent step. This is done using water (either with or without detergent) or with solvents, depending on the type of lubricant used. Cleaning is generally performed in an ultrasonic cleaner unless the specimen is very brittle or composite (risk of phase separation) or unless the sample is metallic (risk of cavitation and oxidation). *Caution*: Ultrasound waves produce heat. In some cases, chemical reagents are used: boiling H_2O_2, nitric acid, potassium dichromate and hydrofluoric acid (*caution*: it attacks oxides), and ammonium difluoride (for glasses). Cleaning efficiency is monitored using an optical microscope.

The sample must be carefully cleaned after the final thinning step to remove any contamination before observation in the transmission electron microscope. It is necessary to make sure that the cleaning method does not change the sample's nature. The choice of technique will be based on the type of analysis or observation to be performed.

Listed below are some final cleaning methods:

- Cleaning in water or solvent without ultrasound waves: The sample is held with a pair of tweezers and immersed into the liquid. It is then dried, either on absorbent paper, air-dried, or, better yet, under an infrared lamp.
- Thermal, argon/oxygen, or oxygen plasma treatment to remove contaminants such as grease or hydrocarbons: Depending on the temperature, the thermal treatment can remove these contaminants or reduce them to an amorphous carbon state. This is an effective method for preventing contamination under the electron beam. Risk: Oxidation of sensitive materials.
- Cathodic electrochemical treatment for removing an oxide layer from metals: This method uses the reducing action of hydrogen. *Risk*: Introducing hydrogen into the structure.
- Chemical cleaning for removing the surface layer: This could be an amorphous layer (by oxidation or implantation via an ion beam), a strain hardening layer, or a contamination layer. Risk: Creating a compound reaction.
- Electropolishing for metals and semiconductors: This is an anodic dissolution that removes a strain-hardening layer. Without risk, except for the possible formation of an oxide layer approximately 3-nm thick.
- Ion beam cleaning. Risk: Ion implantation and surface amorphization.

3.2.2 Procedure

There is always "one" better choice among these three parameters: the quantity of material to polish, how quickly the results are needed, and the final time/price spent to prepare a good sample. Sometimes it is better to spend more time with more steps for a less expensive result. If only one sample is processed, the cost is not very important, but if there are many samples, this obviously would be more expensive.

Depending on the case, several steps to polish the material can be carried out: rough grinding, lapping, pre-polishing, finishing polishing, and chemical, electrochemical, or ionic polishing.

For most materials, it is routine to use two pre-polishing platforms or disks and three polishing disks to end up with a 1,000 grade (18 μm) and two finishing cloths to end up with 1 μm.

The sample must be firmly attached facedown to the lower base of a heavy specimen holder (metal cylinder) either by mechanical locking or by hot or cold gluing with glues or waxes. The specimen holder can be equipped with a tiltable head for correcting the polishing plane. Generally, a microscale can be used to determine how much material is removed.

For rough grinding, the specimen can be held by hand. The lower side of the sample is held against the rotating abrasive disk. Lubrication provided by a liquid flowing on the platform is essential for clearing away debris from wear and in order to reduce friction-induced temperature rise. Water, oil, or special lubricants are generally used.

In the simplest case, the specimen holder sits on the polishing wheel under its own weight or it is held and pressed down by hand.

These techniques use either two-body mechanical action to polish the specimen on a wheel with a fixed abrasive or three-body action when the abrasive is loose between the wheel and the specimen. The friction of the abrasive grains progressively wears away the material.

Polishing starts with large abrasive grains and the grain size is decreased in successive steps. The specimen's position is changed on the polishing wheel in order to prevent the sample from always having the same trajectory on the disk. A large amount of water must be used, but if too much is used, hydroplaning occurs (the sample "floats" on the surface of the water) and the result is ineffective. A set of wheels or cloths can only be used for one type of material.

During all of the steps, especially the finishing step, a stereoscopic microscope or optical microscope must be used to verify polishing quality. In particular, the microscopist must spot scratches caused by the previous polishing step and also see if there are encrusted abrasive grains. In all cases, it is recommended to carefully choose the material used in order to minimize mechanical damage.

The following parameters need to be controlled:

– Sample: Type of chemical bond, hardness, and ductility
– Support: Hard or soft
– Quantity and nature of the abrasive: bound or loose, hardness
– Quantity and nature of the lubricant: helps with polishing
– Penetrating force
– Rotation speed
– Polishing time

Polishing time must be as short as possible at all times, since the longer the polishing time, the higher the temperature and the more mechanical damage is caused.

Each step must be checked under an optical microscope and a step is completed when there are no more scratches or defects from the previous step.

The main defects are as follows:

(1) Rounded sample edges. These occur

- when the sample is not held flat (held by hand or with too light of a support). In this case, either a good polishing guide or a heavy polishing specimen holder that is significantly larger than the sample must be used

- when using a polishing cloth that it is not hard enough. The sample is worn on the edge, pushing back the platform's fibers like on a felt hat. In this case, a woven cloth must be used instead of a flocked or unwoven cloth

(2) Surface roughness caused by structural variations:

- If the sample is embedded the embedding material wears faster because it is not hard enough. For an embedded sample, the hardness of the embedding material must be matched to the sample hardness. For example, reinforcing fibers must be added if necessary or the sample must be placed on a specimen holder with hard edges that wear more slowly but ensures the levelness of the setup without the abrasive wearing the embedding material more quickly than the sample.
- If the sample wears faster, the embedding material is too hard. This happens on cloths where the fibers are too long; they polish the bottom of the cuvette. This is also the case if the cloth is too damp.
- If the sample is a bulk sample presenting reliefs, they are probably due to variations in structural homogeneity. The only solution is to work with a platform of woven cloth and a polishing guide that is harder than the sample.

(3) Creep: This occurs if the material is soft and the force applied during polishing is too great. The solution is to use low pressure and a large quantity of lubricant. The sample must almost float above the polishing platform.

(4) Scratching. This occurs

- in brittle samples. Polishing is carried out with an abrasive that is too hard and too strong, or the polishing cloth is too dry
- if there is a second phase that is more fragile than the first

(5) Porosities. These are observed

- when precipitates are loosened. There is no solution other than to apply a very gentle polishing
- if abrasive grains are embedded in the surface. If the action is continued, they roll on themselves and create a significant cavity. This is frequently tied to the use of cloth fibers, in particular for ductile materials

Surface cleaning, especially the clearing away of abrasive grains, must occur between each step.

Final polishing removes very little material. There must not be any visible scratches; the surface must be shiny and glossy with a mirror polish. This step must be as brief as possible because otherwise over-polishing occurs and there may be rounded edges and holes (abrasive grains embedded in the sample).

3.3 Variants

3.3.1 Vibrating Plate

This technique uses non-rotating metal plates. The plate is generally agitated mechanically or ultrasonically. The specimens are either loose (if they are large enough) or placed on specimen holders to give them some weight and then they are placed directly on the plate. The abrasive is scattered on the plate and can be combined with a lubricant. Specimen movement is random. This technique is used mainly for industrial polishing.

3.3.2 Tripod Polishing

See Chapter 4, Section 3.

3.3.3 Mechanical/Chemical Polishing

Polishing is carried out with a chemical agent such as alkaline colloidal silica or acids on cloths.

3.3.4 Electropolishing

See Section 5.

3.3.5 Chemical Polishing

See Section 6.

3.4 Advantages

These polishing techniques are used to reduce sample thickness and obtain perfectly flat surfaces. They are used to trim down surfaces obtained by sawing and other preliminary sample preparation techniques. Depending on the material properties, the sample thickness can be reduced down to approximately 30 μm.

3.5 Limitations

Given the mechanical action, there is strain hardening over the entire surface for hard materials, mechanical damage for soft and ductile materials, and a thermal effect due to friction. The use of a lubricant is essential for minimizing these effects. Material transport may also occur. All of these defects can be eliminated by other final preparation techniques such as chemical or electrolytic polishing and ion milling.

3.6 Compatible Techniques

Grinding, dimpling, electropolishing, twin-jet electropolishing, full-bath electropolishing, chemical polishing, twin-jet chemical polishing, full-bath chemical polishing, sandwich technique, ion thinning, FIB, and tripod polishing.

3.7 Risks

Inhalation of abrasive dust or sample dust, in particular metals, GaAs (noxiousness), etc.

3.8 Conclusion

Mechanical polishing is a commonly used step for preliminary sample preparation, especially for multiphase metals that are difficult to prepare by chemical or electropolishing. This is also the way to polish cross-sectional samples, except for those prepared using the tripod technique.

4 Dimpling

4.1 Principle

This technique is used to grind a concave impression (polishing in a basin shape) or dimple, thinning the center of a disk to 3 mm in diameter and about 100-μm thick, so that final thinning by other preparation techniques such as electrolytic or chemical polishing or ion milling can then be performed. In most cases, this means producing a scratch-free dimple while limiting mechanical damage. Readers should consult the chapter on polishing ("Methodology", Chapter 2, Section 3) for the technique's protocol and limitations.

This polishing technique employs mechanical abrasion. The friction of the abrasive grains, which are harder than the specimen to be polished, is used to wear down materials. A fluid must be used, serving as a lubricant and coolant.

The technique can be used to polish almost all bulk, compact, and multilayer materials, and even fine particles or porous materials if they are embedded beforehand. They can be multiphase materials and can contain precipitates or segregations. They can also be of any hardness, with the exception of very soft materials.

4.2 Operating Mode

4.2.1 Equipment and Supplies

Commercially available dimplers are used. The device must be placed on a vibration-free flat surface in a clean atmosphere. The purpose is to produce a basin-

shaped grinding to thin the center of the sample while keeping the edges relatively thick.

The sample is a thin slice in the form of a 3-mm-diameter disk around 100-μm thick obtained by mechanical polishing. The slice is stuck to the center of a cylindrical support, and this support is mounted onto an adjustable-speed rotating axle. An arm containing an adjustable-speed rotating grinding wheel made of bronze with phosphorous, steel, etc. is placed perpendicular to the support. The arm is equipped with a counterweight (5–20 g) to regulate the rate of the grinding wheel's penetration into the material. A micrometric sensor is used to constantly measure the depth of abrasion. Some devices are equipped with an automatic system to stop grinding at a chosen depth. A lighting system can be used to monitor the advancement of polishing for transparent samples. Different diameters of grinding wheels are available (5–20 mm) depending on the desired dimple geometry (breadth and depth). The edge of the grinding wheel can be flat or rounded. Spheres of different diameters can also be used.

Thus, there are two perpendicular rotational movements that create the basin-shaped grinding. The grinding wheel is made of metal (e.g., bronze), plastic, or cloth for final polishing. A paste containing the abrasive is placed between the sample and the grinding wheel. The resulting polishing creates a dimple-shaped wear with a surfacing effect (Figs. 2.17 and 2.18).

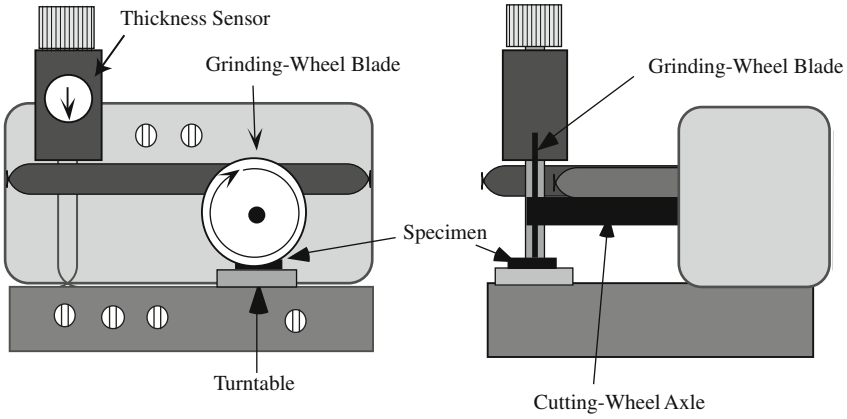

Fig. 2.17 Dimpler diagram

There are many types of abrasives that can be chosen, based on the nature of the material and the finishing quality desired (see Chapter 2, Section 3).

4.2.2 Procedure

This technique employs a three-body mechanical action, because the abrasive is loose between the disk and the sample. The friction of the abrasive grains progressively wears away the material. Readers should consult the chapter on polishing for

Fig. 2.18 The grinding wheel (*vertical*) on the rotating thin slice (*horizontal*)

the technique's protocol and limitations. The abrasives used are generally carbides, nitrates, borides, or diamond.

The sample is a flat disk with parallel faces obtained by mechanical polishing, cut to 3 mm in diameter (grinding, punching, etc.), or is even a cross section prepared using the sandwich technique, variant 1 or 2. It must be less than 100-μm thick. Both faces must be rigorously parallel, as being off by even one degree results in an error of 25 μm of thickness from one side of the sample to the other, resulting in the same uncertainty in the final thickness of the sample center. The mechanical zero of the micrometer is based on the bare sample support placed on the vertical rotating axle.

The sample is hot glued to the sample support, which is placed on the rotating vertical axle. Thermoplastic waxes at low temperatures are used for this. The thickness of the sample and the glue when cooled must be precisely known. It is important to properly center the slice under a stereoscopic microscope. Once the diameter of the grinding wheel is determined, it is placed on the other rotating horizontal axle. The grinding wheel/sample contact point is then perfectly centered using the stereoscopic microscope. Polishing is then started using abrasive grains of the same diameter as those used for the previous polishing step, reducing the grain size in the successive steps. The abrasive is generally a paste containing the lubricant, but it can also be a powder to which a lubricant is added. As with polishing, the sample must be thoroughly cleaned between each step in order to clear away any abrasive grains, otherwise scratches will be produced in the following step.

The depth of the dimple must be checked frequently. After a dimple is made on one face, the sample is then flipped over to create a dimple on the other side.

Normally, it is common to have a thickness of 5–10 μm in the center of the dimple. The depth of the dimple must be measured precisely, penetration force must be minuscule, and the final abrasive grades must be very fine. Finishing is done with a polishing felt or by mechanical/chemical polishing.

For samples that have a coating or surface segregation, a dimple is formed on a single face, preserving the coating on the rear face. Final thinning will be performed on the coated side using finishing techniques (electrochemical, chemical, or ionic thinning).

When applied to coated specimens, this technique can be used to wear down the coating and create a cut for studying its profile, e.g., using X-ray energy-dispersive spectroscopy.

During all of the steps, especially finishing, a stereoscopic microscope or optical microscope must be used to verify polishing quality and in particular to spot the scratches caused by the previous polishing step.

As with polishing, all dimpling steps must be as brief as possible. The parameters to be controlled in order to prevent mechanical damage are rotational speeds of the specimen and the grinding wheel, quantity and type of abrasive and lubricant, penetrating force (grinding wheel axle weight), and polishing time.

4.3 Variants

There are no variants.

4.4 Advantages

This polishing technique is used to obtain regular, surfaced dimples. It is used to thin the center of 3-mm disks, while minimizing mechanical damage. It can be used to obtain electron-transparent surfaces much larger than if the sample had no prior dimpling. The thickness of parallel face slices can be reduced down to 5 μm while maintaining the disk's rigidity, because the edges remain thick. This technique induces little mechanical damage if performed properly and can considerably reduce the final thinning time using other preparation techniques such as chemical, electrochemical, and especially ionic thinning.

4.5 Limitations

Due to the mechanical action of the technique, there is strain hardening for hard materials, mechanical damage for soft and ductile materials, and a thermal effect due to friction. The material used must be chosen carefully in order to minimize mechanical damage. Material transport may also occur. All of these defects can be eliminated by final thinning such as chemical or electrolytic polishing or ion milling.

4.6 Compatible Techniques

Electrolytic polishing, twin-jet electrolytic polishing, full-bath electrolytic polishing, chemical polishing, twin-jet chemical polishing, full-bath chemical polishing, and ion milling.

4.7 Risks

None.

4.8 Conclusion

Dimpling saves considerable time, especially for hard or brittle materials. If final thinning is performed by ion milling, reducing thickness in the area of interest makes it possible to minimize radiation damage tied to milling time.

5 Electropolishing

5.1 Principle

This preliminary preparation technique is used to obtain shiny surfaces on conductive samples polished flat mechanically or made by sawing; it is also used to clear away strain hardening. This strain hardening can come from the use of other preliminary preparation or thinning techniques. Samples of any shape can be polished.

Polishing a conductive body is a full-bath electrochemical-etching technique based on the theory of electrolytic polishing. The viscous layer formed on the sample surface by anodic dissolution results in a differential dissolution rate of the relief, having the effect of removing rough parts and resulting in a flat shiny surface while dissolving the material.

This technique only applies to conductive materials such as metals or semiconductors. They must be bulk, compact, and generally single phased, whether they are brittle or ductile. For certain multiphase materials and/or those containing precipitates or segregations, it is sometimes possible to find polishing conditions.

5.2 Operating Mode

5.2.1 Equipment and Supplies

The equipment consists of an electrical power supply or a potentiostat that regulates the potential (voltage) and therefore the dissolution current. A milliammeter and a

millivoltmeter must also be added. The equipment is inexpensive and commonly used in laboratories. Machines specially dedicated to electropolishing are commercially available. They can be found in specific catalogues for polishing equipment or for electron microscopy equipment. Cables are also needed to connect the electrical power supply to the sample. The anodes and cathodes are the electrodes of the polishing cell. The "anode" sample is connected to the positive, or anodic, pole while the cathodic, or negative, pole is connected to a plate that constitutes the cathode. This must still be larger than the sample (5–10 times larger than the sample). It will be of different compositions depending on the type of metal to be polished (e.g., copper for copper, a stainless steel for steel and ferrous metals, or a platinum grid in most other cases). The setup (see Fig. 2.19) is placed in the polishing cell and immersed in the electrolytic solution. Since it is often necessary to work at low temperatures (there can be significant sample temperature rise up to 393 K), a double-walled cell connected to a cryostat is used to regulate the temperature. A low temperature promotes decreased dissolution currents and increased viscosity of the polishing layer. Polishing conditions include bath temperature, voltage, and polishing time. The cathode material types are provided either with solutions purchased commercially (the composition of the bath is generally a trade secret) or with the solutions cited in reference works. Many electrolyte references are found in transmission electron microscopy, electrochemistry, or electrolytic polishing books as well as in materials handbooks. These baths are provided for categories of materials or in specific cases, e.g., for semiconductors or semiconductor oxides.

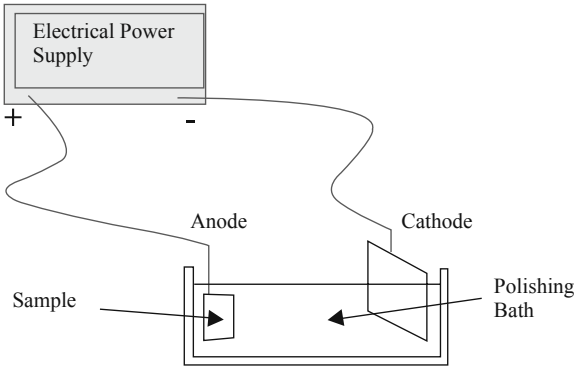

Fig. 2.19 Diagram showing the setup for electropolishing

Solutions are combinations of acids and solvents to which thickeners are added to increase the solution viscosity.

5.2.2 Procedure

The purpose of electropolishing is to make the surface of metals shiny and free from scratches and remove stresses from strain hardening associated with sawing or mechanical polishing. The sample is generally a foil or slice a few square centimeters in area (or less). Only the face of the sample to be treated is in contact with the electrolytic bath. The rest of the sample is completely isolated to eliminate edge

effects. If necessary, the entire rear surface (which should not to be polished) can be covered with a varnish, but this is not mandatory. The sample constituting the anode is then placed facing the cathode, at a distance of approximately several centimeters, in a full bath in the temperature-regulated polishing cell.

The composition of the electrolytic bath and the temperature, current, and voltage (holding potential) conditions are provided in specialized publications, such as the metallurgy handbook. There are often many operating conditions for all conducting or semiconducting materials. *Note*: An "aged" solution is always better than a fresh solution for ferrous metals, but the opposite is true for light metals.

Since polishing parameters vary with cell geometry (electrode dimension, relative position with regard to one another, and bath temperature), it is often necessary to make a current–voltage curve in the electrolytic bath on a test sample. The goal is to precisely determine the position of the electrolytic polishing plateau plate in a specific potential range. This plateau is due to the presence of a viscous film created by dissolution products on the surface of the anode, which presents a high electrical resistance.

To do this, the sample is placed in the cell facing the cathode. Generally, the sample is vertical and slightly tilted, faceup, so the dissolution layer slides and slowly flows downward. In other rare cases, e.g., copper, the sample must be at the bottom of the cell with the face to be polished facing upward and the cathode is placed 1 or 2 cm above it. First, a potential near 0 V is applied. The sample then reaches an equilibrium current value of dissolution. This value is read off with a milliammeter. The value is plotted on graph paper, and then the potential is increased one unit at a time until the electrolytic polishing plateau is reached. In practice, the polishing plateau is between a few volts and as much as 15–20 V, but it can sometimes reach 40 V. Once the curve is traced, a more or less pronounced plateau or inflection point should be visible.

Figure 2.20 shows an example of a perfect polishing curve (curve 1) and a more commonly encountered curve (curve 2). In the cathodic domain, there is hydrogen release at low potentials, whereas in the anodic domain, there is oxygen release at high potentials. The plateau represents a potential zone where there is a viscous layer limiting the current and passivating the metal. At the beginning of the plateau, there is a risk of sputter, and at the end, there is a risk of pitting. On the right-hand

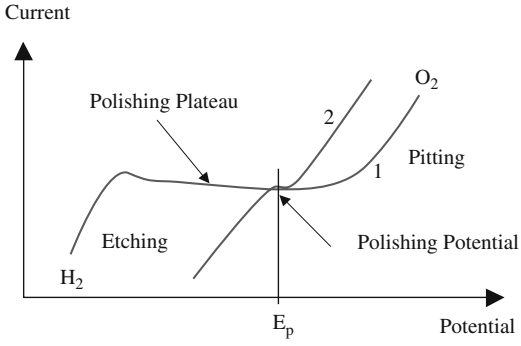

Fig. 2.20 Example of a current–voltage curve

side of the plateau, there is an electrical voltage that gives rise to a maximum and therefore effective resistance. The proper polishing voltage value (potential) is close to the upper third of the plateau. Beyond that, the layer is destroyed and the anode is attacked irregularly by holes on the surface. Sometimes the plateau is wide and it is hard to find the right potential. The effects of this can be limited by making an ohmic drop correction. The cooling temperature of the bath can be varied in order to improve polishing, because, in particular, this reduces current densities and increases polishing layer viscosity.

It is possible to work in batches when the polishing conditions are found on the test sample. The sample is placed under voltage in the cell for polishing. The viscous layer formed on the sample surface results in differential dissolution of the relief, which has the effect of removing rough parts, resulting in a flat shiny surface while the material dissolves. The sample is removed from the bath under voltage and washed immediately. The varnish is then dissolved if necessary. The sample is rinsed and then dried (Figs. 2.21 and 2.22).

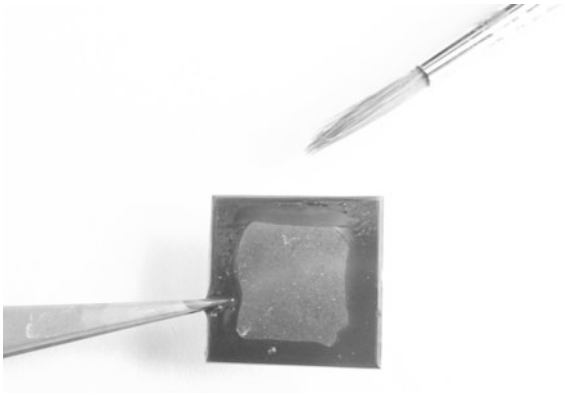

Fig. 2.21 Varnish protection on the edges of the specimen

5.3 Variants

Brush polishing or electromechanical polishing. This variant consists of laying the specimen (anode) flat and passing a metal brush (cathode) containing the electrolyte over the zone to be polished. This technique can be used to polish a specific area of the sample, which can be a part of a larger piece.

5.4 Advantages

This technique can be used to obtain a bright surface polish regardless of the hardness of the metal or semiconductor. The resulting slice is thoroughly polished and free of surface roughness. The structure to be observed is without strain hardening or stress deformations. There is no risk of material transport during polishing.

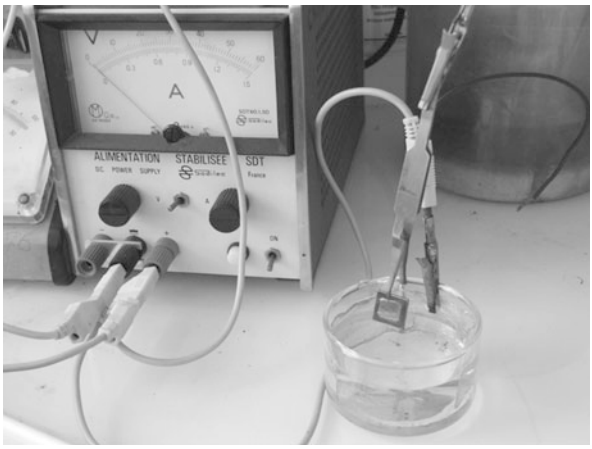

Fig. 2.22 Electrochemical equipment

5.5 Limitations

Electropolishing does not apply to non-conductive materials. The difficulty for conductive materials is finding the electrolytic bath, potential conditions, current, and adequate temperature. This is especially true for complex or exotic materials. There may be a significant temperature rise of the samples up to 393 K. In the case of multiphase materials, there is a risk of selective dissolution or loosening, e.g., on precipitates. In all cases, there may be dissolution products remaining, such as oxides coming from the surface release of oxygen, if the bath is not appropriate or if the conditions for proper polishing are not met, but this is rare and only concerns a very thin contamination layer (1–2 nm) that will be cleared away by the subsequent preparation steps. Generally, there is contamination from elements due to the bath, which is detrimental for X-ray or electron energy loss analysis. In the case of alloys, there can even be enrichment by one or more elements on the surface by the re-deposition of an element. We then must employ ion milling to clean the surface. This is ideal because the milling time is very short and there is little risk of implantation damage.

5.6 Compatible Techniques

Ultrasonic grinding, twin-jet electrolytic thinning, full-bath electrolytic thinning, ion milling, FIB, and tripod polishing.

5.7 Risks

The use of chemicals may create risks of chemical burns and inhalation of noxious aerosols. Attention must be paid to the dangers of electrolytes depending on their

composition and concentration (e.g., chemical risk or even risk of explosion with perchlorate).

5.8 Conclusion

This technique is generally used for single-phase conducting materials, regardless of their hardness. It generates almost no preparation artifacts.

6 Chemical Polishing

6.1 Principle

The purpose of this preliminary preparation technique is to achieve surface etching with a polishing effect. This technique is used to obtain level surfaces on materials polished flat mechanically or made by sawing. It is also used to clear away strain hardening. This strain hardening can come from the use of other preliminary preparation or thinning techniques. Samples of any shape can be polished. This is often the final step for cleaning surface contamination and removing damage caused by mechanical stresses.

This is a full-bath dissolution technique based on the polishing theory. The layer formed on the sample surface leads to a differential dissolution of the relief, which has the effect of removing bumps, resulting in a flat shiny surface. For some materials, polishing is not achieved because the etching is not differential, but the hardened part of the sample is still removed.

This technique applies to all types of reactive materials. They must be bulk, compact, and generally single phased, whether they are brittle or ductile. For certain multiphase materials and/or those containing precipitates or segregations, it is sometimes possible to find the right polishing conditions. Obviously, noble metal samples (platinum, gold, etc.) present difficulties, but there are solutions. It is practically impossible to prepare polymer (Teflon) or organic materials using chemical polishing.

6.2 Operating Mode

6.2.1 Equipment and Supplies

The equipment consists of a chemical bath in an adjustable-temperature glass or Teflon vessel. The sample is placed in a full bath in a beaker, which is either temperature controlled or not. Only the face of the sample to be treated is in contact with the chemical bath. The process is monitored using a stereoscopic microscope. The sample is laid at the bottom of the beaker (or etching cell) or held below the surface in the chemical bath using tweezers (Fig. 2.23). The composition of the etching solution depends on the material's chemical composition, but in general the

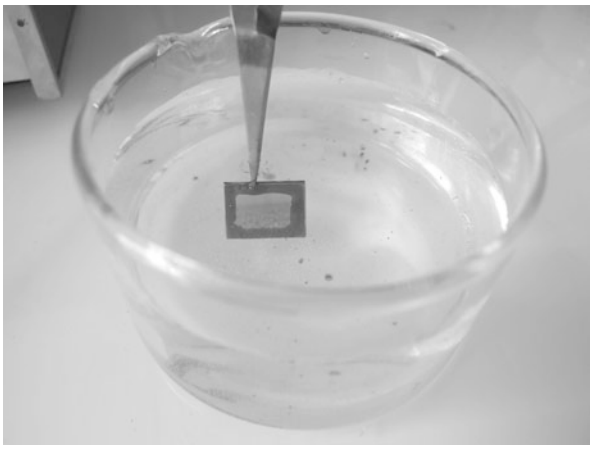

Fig. 2.23 Chemical etching cell

solutions are acids or alkalines. Compositions are provided in specialized works or through knowledge of chemistry.

6.2.2 Procedure

The sample is generally a foil of a few square centimeters or a slice obtained using sawing or mechanical polishing. However, we also handle slices (3 mm) that are pre-thinned by dimpling in order to remove strain-hardening layers or also slices that are thinned using other techniques in order to remove contamination layers. The sample is immersed in a bath in a temperature-controlled beaker. It reaches a balance potential as a function of the solution's balance potential. Only the face of the sample to be treated is in contact with the chemical solution; the other part of the sample is protected using a varnish. The sample is etched, rinsed with water or another solvent, and then dried.

6.3 Variants

The variant is a mechanical/chemical technique: etching chemicals are used at the same time as the sample surface is rubbed using a polishing cloth or wool. This is the case with cerium oxide or colloidal silica, which are chemical reagents. Nevertheless, there is a combination of mechanical and chemical effects in colloidal silica, because the silica solution used is often alkaline (pH 9).

6.4 Advantages

This technique can be used to obtain a good-quality polish regardless of material hardness. The resulting slice is polished and free of surface roughness. The structure

to be observed is free of strain hardening or stress deformations. There is no risk of material transport during polishing.

6.5 Limitations

The difficulty lies in finding the right bath and adequate temperature, especially for complex or exotic materials. For multiphase materials, there is a risk of selective dissolution, e.g., with precipitates. There is also a risk of revealing dense atomic planes having low crystallographic indices.

In all of the cases, there may be dissolution products (oxide, sulfur, etc.) left behind on the surface if the chemical solution is not suitable or if the right polishing conditions are not met, but this is rare and only concerns a very thin contamination layer that will be cleared away by the subsequent preparation steps.

6.6 Compatible Techniques

Ultrasonic grinding, twin-jet electrolytic thinning, full-bath electrolytic thinning, ion thinning, FIB, and tripod polishing.

6.7 Risks

The use of chemicals may create risks of chemical burns and inhalation of noxious aerosols, as well as a hazardous explosion in some cases.

6.8 Conclusion

This technique is used for all single-phase materials, regardless of their hardness. It generates practically no preparation artifacts.

7 Sandwich Technique

7.1 Principle

Materials that must be observed in cross section are usually multilayer materials. Different types of analysis, such as measuring layer thickness, interface, or composition gradient observation, require observation parallel to the layers. Bulk materials, such as a material that has undergone an implantation (of ions, particles, etc.), also require cross-sectional preparation to be able to observe the implantation depth and the density of the ions or particles. In order to be able to carry out this type of observation, the sample must be prepared in cross section. Preparing the material for cross section without protecting the surface results in the loss of surface layers that will be abraded during ultrasonic cutting, mechanical pre-polishing, dimpling, ion milling,

or the tripod technique. Pre-preparation of the sandwich, which consists of making a stack of at least two strips of the sample stuck face-to-face using a polymer resin, helps to protect the surface layers during the thinning process. Furthermore, the quantity of observable material will be doubled or multiplied depending on the number of strips put together. Thus, different samples having similar mechanical behaviors can be prepared in a single preparation. The stack also increases the size of the sample to be handled.

The nature of the material or type of preparation to be performed may require the sandwich to either be embedded in a metal cylinder in order to reinforce it or it may be mounted in a special ring that squeezes the samples from the side. These procedures are described with two variants.

Sandwich pre-preparation is applicable to any type of bulk or multilayer, compact material, including metal, semiconductor, ceramic, mineral, or mixed–composite materials.

7.2 Operating Mode

7.2.1 Equipment and Supplies

The equipment used for this preparation consists of a small press that compresses the sample in order to obtain a very thin glue film (Fig. 2.24). The plates above and below the place where the sample is laid are covered with a Teflon strip to prevent excess glue oozing from the edges of the sandwich from sticking permanently to the metal parts of the press. Depending on the type of glue (polymer) used, polymerization will require placing the sandwich–press assembly onto a hot plate at up to

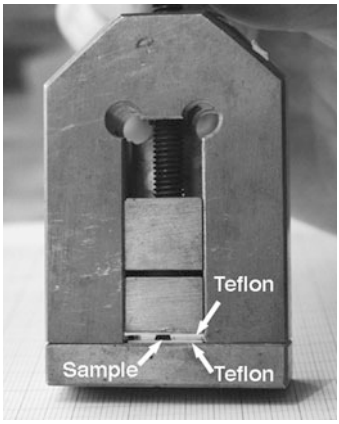

Fig. 2.24 Sample compressed in the press between two Teflon films

approximately 373 K or under an ultraviolet lamp. A heat-sensitive material must be glued with a polymer that hardens at room temperature.

7.2.2 Procedure

Cutting the Sample

The sample must be reduced to the required dimensions. The cutting method – sawing, grinding, cleavage, EDM (electrical discharge machining), or otherwise – is determined by the material type. The final dimensions of the sample are a function of several parameters:

- The thinning technique or variant that will be used afterward
- Sandwich preparation type (standard, variant 1, and variant 2), selected based on the thinning technique and material type
- In some cases, the amount of material available

For a preparation using the wedge-tripod method, the dimensions are approximately 1.7-mm wide × 5–10 mm long, with thickness varying between 0.2 and 1 mm (Fig. 2.25). Final thickness is often determined by the sample thickness. For example, substrates of semiconductors such as silicon or gallium arsenide have thicknesses of 350 or 500 μm, and it is not necessary to reduce this thickness. Reducing sample thickness can be done by sawing or by mechanical or chemical polishing of the substrate.

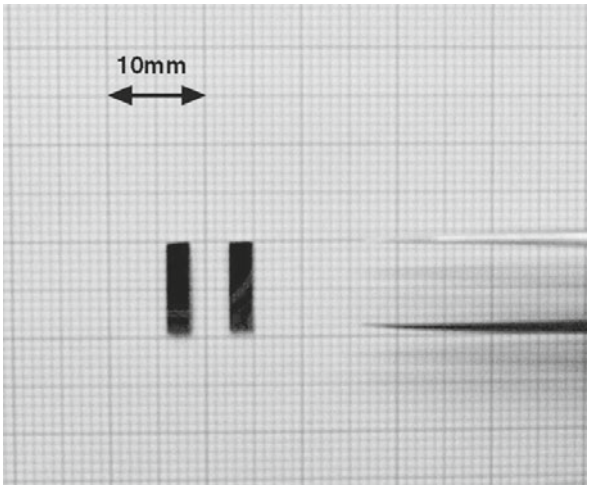

Fig. 2.25 Cleaved or sawn strips

After cutting, the strips are carefully cleaned in successive baths of solvents suitable for the particularities of the material. The surfaces to be glued must be thoroughly cleaned in order to provide proper adhesion.

Gluing

Several types of polymer, often called "glue" for such preparation techniques, can be used for gluing. However, they must meet several decisive criteria, in particular, they must be

- stable under the electron beam and under the ion beam, if this preparation is required
- of significant hardness after polymerization

Materials that must be prepared in cross section are generally harder than a hardened polymer. This difference in hardness can, during mechanical or ionic abrasion, cause faster abrasion of the polymer (glue) than the material. Consequently, surface layers are lost. A hardness as close as possible to the hardness of the material yields the best results.

Generally a two-part epoxy resin is used, with a low viscosity if possible. Epoxies are relatively stable under the beam and can become very hard after polymerization. EPOTECH 353ND (also available under the brand name G1) and M Bond can be used for polymerization above room temperature, and Araldit can be used for polymerization at room temperature. Any other polymer with the same properties would also suffice. Araldit is a highly viscous epoxy resin, making it difficult to obtain a very thin film. It is also hard to mix without introducing air bubbles that will remain trapped in the glue. The glue must be vacuum degassed before use to eliminate air bubbles introduced when the components are mixed. Residual air bubbles in the film lead to lost surface layers that break during preparation. Glue should be used only when necessary.

EPOTECH 353ND has the advantage of liquefying at around 333 K, before polymerizing, which enables the formation of a very thin film. It is essential to get a glue film as thin as possible, around a few hundred nanometers, for the difference in hardness between the sample and the glue to be "imperceptible." In Fig. 2.26, we can see that the glue line is not visible after polishing using a 3-μm-grade abrasive. It appears only after polishing using a 1-μm-grade abrasive. The thickness of the glue is adequate.

A bit of glue is spread in a thin film over a clean, flat surface, e.g., on a glass slide or smooth piece of paper.

The strips are placed one by one into the glue film, with the surface to be protected facing the glue (Fig. 2.27).

The two coated surfaces are placed one on top of the other, face-to-face, forming a sample/glue/sample sandwich (Fig. 2.28). The sandwich is placed in the press and

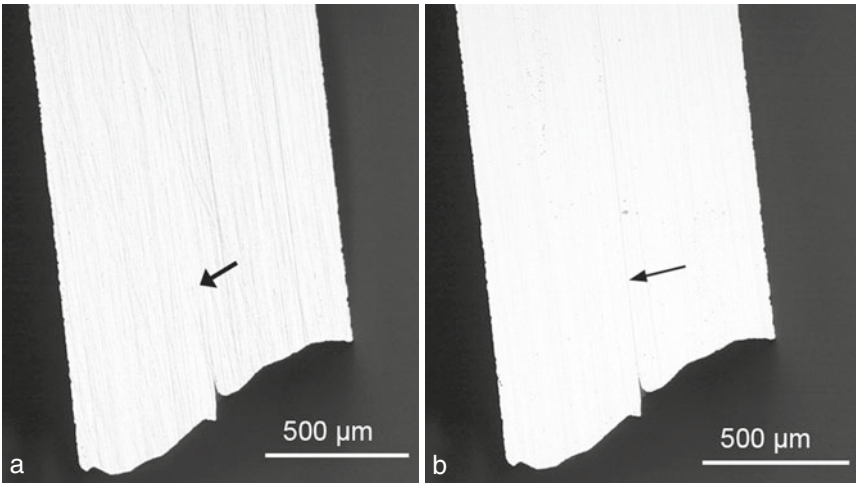

Fig. 2.26 **a**, **b** The *arrows* indicate the location of the glue line. This is not visible after polishing with 3-µm diamond abrasive and appears only after polishing with diamond of a 1-µm grade

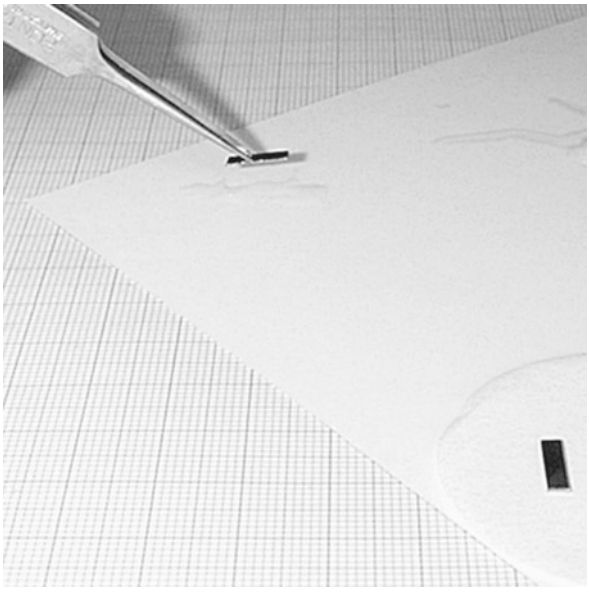

Fig. 2.27 The strips are placed down one by one on the glue film and then mounted one on top of the other

approximately 250–300 g of pressure is applied in order to make a very thin glue layer. The sandwich is then left at room temperature or placed onto a hot plate or a UV lamp for polymerization above room temperature.

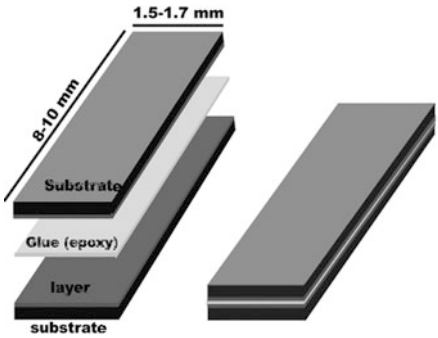

Fig. 2.28 Diagram of a multilayer sample prepared using the sandwich technique for tripod thinning (1.5–1.7 mm wide)

Fig. 2.29 Sandwich cut for tripod polishing or for variant 2

For tripod thinning, the sandwich can be used either as is for polishing the first face or it can be cut into slices approximately 600-µm thick (Fig. 2.29), preferably by abrasive wire sawing.

Several sample strips can be stacked one on top of the other in order to be able to grind the stack perpendicularly. The diameter of the grinding tool is 3 mm, so the stack must be thicker than that in order to grind it into a disk.

7.3 Variants

Both variants described below are generally used to consolidate a sandwich that must be thinned using mechanical pre-polishing (Section 3) before dimpling (Section 4) and/or ion milling (Chapter 3, Section 5).

In variant 1, the sandwich is inserted into a hollow metal tube. In variant 2, a 3-mm-diameter ductile metal ring containing two crossbars approximately 1.5 mm apart is used. The two bars will be deformed as the sandwich is pressed.

It should be noted that the dimensions (length, width, and thickness) are for informational purposes and should be adapted to the dimensions of the supports available.

7.3.1 Variant 1

The sandwich is prepared using the procedure just described, but the sample dimensions are approximately 2.5-mm wide, from 8- to 10-mm long, and approximately 0.5-mm thick (for a strip). The sandwich must be covered with glue and then inserted into a metal or ceramic cylinder that is hollow in the middle (Fig. 2.30c). This setup slides into another 3-mm-diameter hollow cylinder (Fig. 2.30e). One of

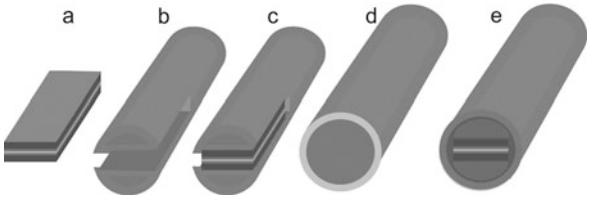

Fig. 2.30 Diagram showing the sandwich going into the cylinder: (**a**) sandwich; (**b**) hollow cylinder; (**c**) sandwich inserted into hollow cylinder; (**d**) 3-mm-diameter hollow cylinder; and (**e**) final setup

the ends must be plugged so as to prevent glue from flowing from this end. The sandwich is then placed either vertically onto a hot plate or under a UV lamp for polymerization above room temperature. For heat-sensitive materials, the setup is left at room temperature. To keep the setup vertical, we can use a Teflon support (it does not stick to the resin) that is hollowed out in the center. The hole will be slightly shorter in depth than the total length of the sample support setup and will have a diameter greater than 3 mm, so that the setup can be removed after polymerization.

Lastly, we cut the sample by sawing a 600- to 800-μm thick section in which the sample is centered, in order to be able to use the mechanical polishing (Chapter 2, Section 3), dimpling (Chapter 2, Section 4), and/or ion bombardment technique (Chapter 2, Section 5).

7.3.2 Variant 2

This technique is used, among others, to compensate for overly poor adherence between the sample surface and the glue. Since the sample is in contact with the metal bars, thermal and electric conductivities are improved.

The material used to fabricate the ring must be ductile enough to deform, but it must also be resistant to mechanical or ionic abrasion. Metals such as copper or aluminum are not suitable.

The sandwich is prepared using the procedure just described, but the sample dimensions are approximately 2.5-mm wide, from 8- to 10-mm long, and approximately 0.5-mm thick (for a strip). Thickness is dependant on the distance between the two cross-bars. Remember that once the sandwich is made, its final thickness will be double.

Under the stereoscopic microscope, the sandwich is placed between the two bars (Fig. 2.31); using two tools, e.g., two small screwdrivers, both bars are simultaneously deformed until they hold the sandwich under pressure (Fig. 2.32). A 3-mm drop of liquid epoxy resin is placed on a Teflon support. The ring-sample setup is placed on the resin drop. The resin will fill openings by capillary action (Fig. 2.32) and the ring-sandwich setup will settle by gravity into the resin drop, until there is just a thin glue film between the Teflon and the ring-sandwich.

The sample-Teflon-support setup is then placed either onto a hot plate or under a UV lamp to polymerize the resin.

Fig. 2.31 Diagram of the sandwich inserted into the metal ring

Fig. 2.32 Diagram of the sandwich pressed by the *cross-bars* and then embedded. The *dark grey color* represents the embedding resin

For mechanical polishing, the side to be abraded first is the unlevel side (milled) and the sandwich-ring setup will be stuck on the flat face.

For this technique, Araldit or any other viscous polymer cannot be used.

7.4 Advantages

This technique is used to protect the surface layers of a material during the various pre-preparation and mechanical or ion-thinning techniques. Sticking at least two pieces of sample against one another multiplies the quantity of observable material and also helps to prepare samples of similar mechanical behavior in a single operation.

7.5 Limitations

For hard and very hard materials, the glue is always softer than the materials. It wears away faster than the sample during subsequent mechanical polishing or ion-milling techniques, which can result in lost surface layers. This effect is greater as the film thickens (Fig. 2.33).

7.6 Compatible Techniques

The sandwich can subsequently be prepared using ultrasonic grinding, mechanical pre-polishing, dimpling, or ion milling and tripod techniques.

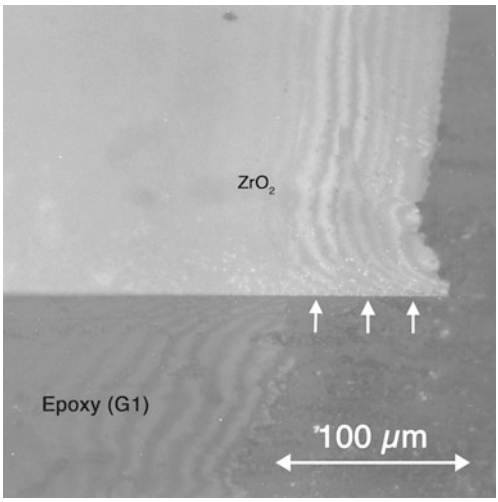

Fig. 2.33 Sample of ZrO_2 in which particles have been implanted on the surface (*white dots*). In this example, the sample was not prepared in a sandwich. A glue film was spread on the surface of the material for cross-sectional wedge preparation. The glue wears away faster than the sample and does not protect the surface of the material, as indicated by the *arrows*. This surface is then abraded by mechanical polishing; there is loss of surface material

7.7 Risks

It is necessary to protect against toxic vapors if solvents are used.

7.8 Conclusion

The sandwich technique is used to obtain a cross section of a multilayer material for the tripod polishing and ion milling preparation techniques. It is a special embedding technique, compared to classic embedding used in ultramicrotomy.

8 Embedding

8.1 Principle

To prepare thin slices of small samples, generally fine particles, using any other method than the fine particle dispersion method (Chapter 5, Section 1) or FIB thinning in certain cases (Chapter 3, Section 6), it is necessary to produce a sample that is a few millimeters across so it can be handled during preparation. These small materials can be in the form of powders, small plates, fibers, small threads, etc.

Conversely, certain large materials that are too soft or brittle must also be embedded to reduce their size. Embedding is also used for mixed samples (e.g., multilayer materials) in order to increase the sample's cohesion.

The embedding preliminary preparation consists of placing the powder or sample into a mold and immersing it into a liquid resin that is then hardened by polymerization. This method is mainly used for the ultramicrotomy technique (Chapter 4, Section 4). It is also used to protect the surface of a small sample or prepare powders using mechanical polishing, dimpling, ion milling, or the tripod polishing techniques.

It applies to metals, semiconductors, ceramics, minerals, polymers, organic materials, and mixed–composite, single-phase, or multiphase materials. They can be bulk, multilayer, or compact materials or fine particles. Porous materials are embedded after being infiltrated with resin to eliminate the gaseous or liquid phase. They are prepared using the substitution–infiltration–embedding method.

8.2 Operating Mode

8.2.1 Equipment and Supplies

Embedding molds are needed for the preparation. They come in a variety of shapes and are made of various materials. The shape is chosen based on the material organization or on the technique to be used. For preparations using ultramicrotomy, the final dimensions after embedding must be compatible with the dimensions of the ultramicrotomy sample supports. The chemical composition of the mold is chosen based on the chemical composition of the resin. There must not be any chemical interaction between the resin and the mold, which might change the resin's properties or create adherence between the mold and the resin. Notably, plastic molds have reactivity with acrylic resins. The most universal molds are made of polyethylene, Teflon, or silicone.

There are two types of mold. Gel-type molds cannot be used to select the sample disposition; it is random. Flat molds allow the sample to be oriented.

The resin is degassed, either before or after embedding, in a vacuum chamber, e.g., a simple rigid plastic container connected to a vacuum pump or a chamber containing an ejector connected to compressed air. This is especially necessary if the embedding resin is confined during polymerization, otherwise the air bubbles evaporate easily.

A centrifuge can be used to precipitate and concentrate fine particles.

Cold embedding is performed in a freezer or refrigerator at 274 K, or better still in a temperature-controlled, adaptable cold chamber.

Polymerization requires either a sterilizer capable of reaching up to 373 K or a UV lamp with a wavelength of less than 315 nm.

8.2.2 Procedure

This technique is performed on a thoroughly clean, dry sample. All surface residues, such as debris, dust, solvent residues, or grease, will interfere with the proper adhesion of the resin to the sample.

Preparing the Embedding Resin

The first step is to choose an embedding resin that is an epoxy or acrylic polymer.

Embedding polymers are mixtures in the liquid state, more or less viscous, that harden through polymerization. Polymerization is a union of several molecules of a compound, forming a single molecule. Once polymerization is carried out, the polymer is of a variable hardness, depending on the type of polymer or on the percentage of the mixture of the compounds. There is no chemical reaction between the resin and the material.

The embedding resin is selected based on several parameters essential to the preparation and observation of samples in transmission electron microscopy (see "Methodology", Chapter 4).

The choice of resin depends on the sample, the technique used for making the thin slice (cutting or abrasion), and on the type of analysis to be carried out.

Highly viscous resins must be liquefied at higher temperatures before mixing, generally at 333 K, in order to enable the sampling of the exact quantity of each component. However, most often samples are taken at room temperature.

In order to ensure maximum fluidity, the resin components should be mixed right at the time of use

- just before the degassing phase if this cannot be done at the same time as embedding (very brittle or low-density sample that could rise to the top when the air is removed)
- just before the embedding phase if degassing and embedding can be performed at the same time

Some resins (Araldit, EM Bed, Spurr, etc.) can be used to obtain different hardness gradients after polymerization (soft, medium, and hard) by changing the proportions of the different components. The hardness of the polymerized resin must be as close as possible to the sample hardness in order to present similar mechanical properties.

The mixture will contain one or more monomers, one or more hardeners, and an accelerator. The mixture must be stirred immediately in order to ensure proper mixing of the different components. This mixture will be kept only for a few hours because it will thicken quickly and start to polymerize at room temperature.

Commercially available products are often pre-mixed and just require the accelerator to be added to trigger the polymerization reaction.

Bulk or Multilayer Material

For ultramicrotomy, bulk or multilayer materials should be smaller than 1 mm^2 in the cutting area. In order to avoid having to perform embedding twice, there are several options:

(1) The thin sample (multilayer, polymer films, etc.) is cut into a triangle measuring a few millimeters at its base (Fig. 2.34a). An incision, a few millimeters long, is

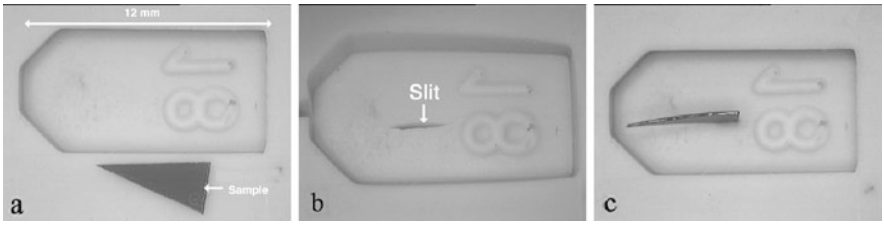

Fig. 2.34 **a, b, c** Steps to embed thin film or multilayer material

made in the bottom of a flat mold using a razor blade (Fig. 2.34b). The sample is then placed vertically in this slit for support (Fig. 2.34c). The resin is then poured over it (under vacuum).

(2) The sample can be raised over the mold by placing it or sticking it to a pedestal that, for example, can be either a piece of metal, paper, or polymerized resin. Attention must be paid so that the end to be cut is placed outside the pedestal.

One technique for very small samples, around 100 μm in size, consists of sticking the end of the sample that is opposite from the end to be cut onto an eyebrow lash, a whisker, or a very fine stiff wire. This setup is then stuck to a pedestal to enable the sample to be totally embedded (Fig. 2.35).

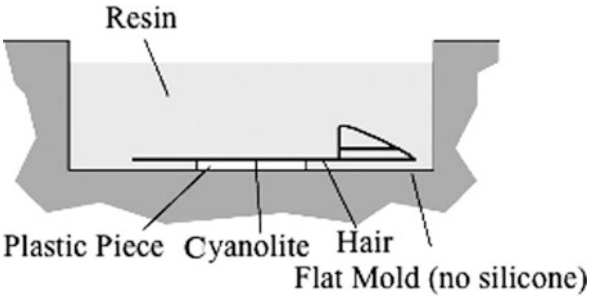

Fig. 2.35 Embedding a bulk material of very small size, stuck to a support (hair), then stuck to another support (paper), before carrying out embedding (according to H. Gnaegi, Diatome)

(3) Hard polymer materials of large dimensions can be cut without the need for embedding. They are reduced to a size suitable for the ultramicrotome′s specimen holder; one end is cut into a small pyramidal shape and the top of the pyramid can be cut using the ultramicrotome.

For other preparations (mechanical polishing, dimpling, ion milling, or tripod polishing), the quantity of resin must be as small as possible compared to the quantity of material in order to prevent selective etching between the resin and the material during the different abrasion modes.

Powders, Small Plates, Fibers, etc. (for a Particular Orientation, See Double Embedding Variant)

The powder can be placed at the bottom of a capsule (Fig. 2.36a) and the resin is poured over it. For polymerization, the capsule is kept vertical.

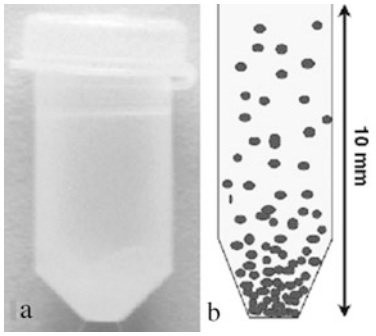

Fig. 2.36 (**a**) Embedding capsule and (**b**) particles embedded in resin

For low-density powders, the resin should be slightly viscous and degassed before embedding in order to prevent the powder from rising to the surface during polymerization.

For ultramicrotomy, a small quantity of powder must be used, but there must be a good grain density in the cutting area. For mechanical or ionic techniques, it is necessary to make sure the quantity of resin is as small as possible compared to the quantity of material, in order to minimize selective abrasion.

For preparation using the ultramicrotomy method, the resulting block (Fig. 2.36b) is cut to a pyramidal shape, whose surface area is no larger than 1 mm^2. For mechanical or ionic thinning techniques, it is necessary to saw a slice approximately 500-μm thick before proceeding with polishing.

Special Embedding Techniques for Fine Particles

Thin Film Embedding: This embedding technique can be useful for powders with a maximum diameter of approximately 30 μm that must be prepared using ion milling techniques. It is used to avoid the mechanical polishing step.

The powder is mixed with an epoxy resin, such as EPOTECH 353ND (G1), and then the mixture is spread out in a thin film on a rigid support (glass slide) covered with a Teflon film. In this step, one or more 3-mm support washers can be incorporated, and then the film is covered with another rigid support that is also covered with a Teflon film. This setup can be inserted in a press or a weight can be placed on it in order to produce a thin film. Polymerization occurs either on a hot plate or in a sterilizer. Next, the film is cut either around the washer or at a diameter of 3 mm using a razor blade. Film prepared without a washer must then be stuck to a 3-mm support.

This preparation is delicate. The film is not of a constant thickness and there may be air bubbles left in the resin. It should be tried if there is little time to prepare the sample.

Sandwich Embedding: This embedding technique is used especially for the tripod polishing technique in order to polish powders, fibers, small plates, etc., with a beveled edge (Fig. 2.37).

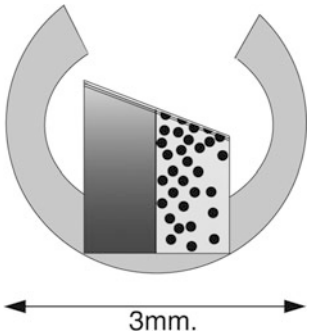

Fig. 2.37 Diagram of a powder embedded in resin, spread out on a silicon support (*at left*) prepared in cross section using the tripod polishing method, then mounted onto a partially cut (to allow ion milling if needed) 3-mm support washer

The powder is mixed with resin and then the mixture is spread out on a silicon substrate. A silicon small plate may (or may not) be added onto the powder–epoxy mixture in order to produce a sandwich. This is all inserted into a small press during polymerization. The pressure applied will be low in order to maintain a relatively thick powder–epoxy film. After polymerization, the setup is cut for cross-sectional preparation.

8.3 Variants

8.3.1 Double Embedding Variant

These double embedding variants are used when a specific sample orientation is needed. First, we cut a small parallelepiped from the block made after an initial embedding, and then it is repositioned according to a preferential orientation during the second embedding.

Fibers, Small Plates, and Orientation for Preparation in Cross Section

The resin is poured halfway to the top of the embedding mold and is then polymerized. Using a fine cutting instrument (razor blade), a narrow crack is made in the center of the resin into which the fibers are dispersed, orienting themselves preferentially lengthwise in the bottom of the crack. The fibers are covered with resin and is allowed to polymerize.

8.3.2 Extraction Variant

This variant is used to extract surface particles from a substrate using double embedding (Fig. 2.38).

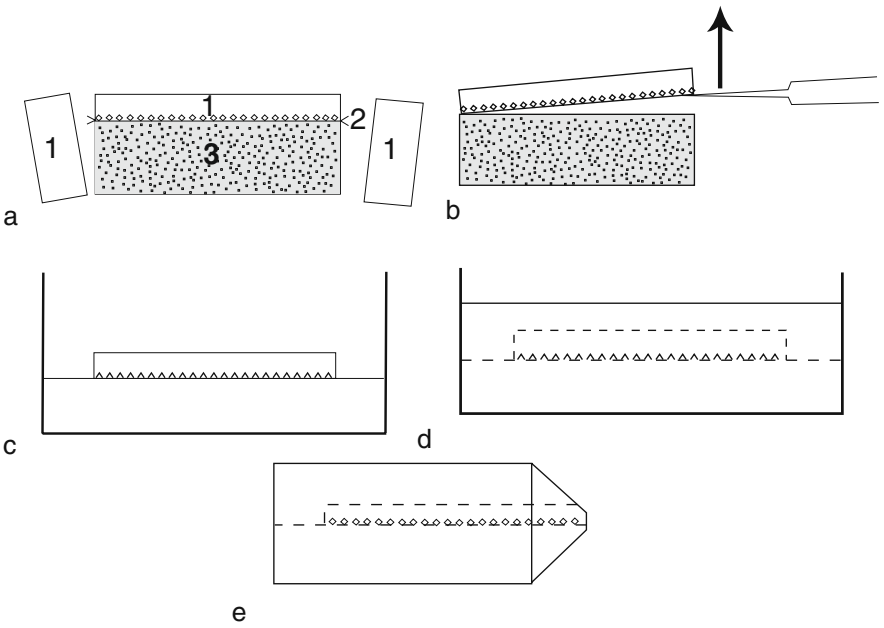

Fig. 2.38 **a** The sample in the form of a rectangular block is embedded in an epoxy resin (1); the altered layer (2) to be preserved can be found on the substrate (3). Once polymerization has occurred, the sides of the sample can be removed. **b** The altered layer of the substrate can be lifted off by inserting a tip at the sample–substrate interface. **c** The removed film is then placed on a base made of the same polymerized resin. **d** The second embedding can be carried out. **e** After polymerization of the second embedding, the sample is ready to carry out cross section by ultramicrotomy

The first embedding is used to extract the particles, and the second embedding serves to protect the extracted surface and orient it.

For example, this variant is used for studies on nuclear waste storage materials to observe altered layers on the surface of nuclear glasses before obtaining ultramicrotome sections (Fig. 2.39).

8.3.3 Variant in Specific Media

For cold preparation techniques (cryo-ultramicrotomy), embedding is performed in specific media that harden by freezing (gelatin, complex sugars, etc.).

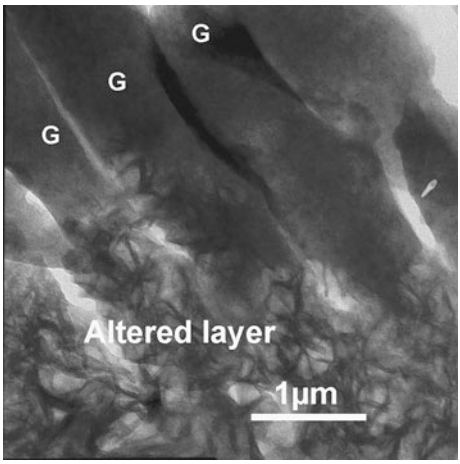

Fig. 2.39 TEM image showing a thin section of a basaltic glass altered in seawater on an unaltered glass substrate (G). The altered layer is composed of the palagonite mineral phase. The sample composed of the substrate (unaltered glass) + altered layer was first embedded in resin. Then after polymerization, we extracted the altered layer, which was embedded a second time by orienting it so as to produce a cross section using ultramicrotomy. The double embedding technique is used to obtain the substrate–altered layer interface (*J.L. Crovisier, EOST, Strasbourg*)

8.4 Advantages

This preliminary preparation is used to maintain cohesion in a multiphase material and to obtain a compact block of very small, fine particles. The fragile surfaces of the material are protected during the process of cutting with an ultramicrotome, or during mechanical or ionic preparations. This technique enables selecting a preferential orientation of the cutting plane by positioning the sample inside the block (cross-sectional or lengthwise cut).

8.5 Limitations

Organic resins can react with the material. The temperature or UV radiation used for polymerization of resins can also change the material's structure. A difference in hardness between the material and the resin can produce a resin-sample separation during ultramicrotomy cutting. The consequences can be

– loss of the sample, which can sink to the bottom of the tank containing the water on which the sections should float so they can be picked up and laid down on a carbon-coated support grid and
– inability to pick up fine particles dispersed on the water surface

8.6 Compatible Techniques

Sawing, mechanical polishing, dimpling, ion milling, FIB, tripod polishing, ultramicrotomy, and cryo-ultramicrotomy

8.7 Risks

There are risks related to carcinogenic chemicals (epoxy or acrylic resins) or organic solvents (1,2-propane, etc.).

8.8 Conclusion

This is a preliminary preparation often used for dry and/or fine particle samples. This method is especially used for the ultramicrotomy technique. For porous or hydrated materials, the substitution–infiltration–embedding at room temperature technique must be used.

9 Substitution–Infiltration–Embedding at Room Temperature

9.1 Principle

This preliminary preparation technique involves creating a hard, workable block in order to cut it and prepare a thin section. The sample can be either a porous material or one containing a liquid phase. This technique, used to homogenously fill in the gaps of porous materials and also to replace the liquid phase with a resin, is mainly used for the ultramicrotomy technique (Chapter 4, Section 4). It is important to make a distinction between embedding and infiltration–embedding. Embedding (Chapter 2, Section 8) consists of homogeneously surrounding a specimen with a polymer resin in order to hold or orientate it more easily. In infiltration–embedding, the polymer resin surrounds (as with embedding) and also penetrates the sample as homogeneously as possible. In the second case, the technique is also used to hold the sample and set its orientation in the resin block.

The infiltration technique is performed after dehydration. The solvent used (alcohol, acetone, etc.) replaces the air or the liquid phase; this solvent will gradually be replaced by a resin before polymerization is carried out.

This technique can be applied to biological materials, polymers, minerals, soft metals, fine particles, etc.

9.2 Operating Mode

9.2.1 Equipment and Supplies

A tank, UV lamp, vacuum chamber, centrifuge, stirrer, and special molds are required.

9.2.2 Procedure

This technique may follow the chemical fixation technique (see Chapter 2, Section 11).

It contains four steps:

1. Dehydration: substituting the air or liquid solution (usually water) with an organic solvent
2. Infiltration: substituting the solvent with a liquid resin
3. Embedding: surrounding the sample with resin
4. Polymerization of the resin

Substitutions must be gradual and complete.

Dehydration

This step is performed on hydrated biological samples that have been chemically fixed. In order to protect cells or tissues during dehydration, a low melting point (LMP) agar solution of 2% in water can be used to infiltrate the sample so it can be easily handled and cut into small pieces of approximately 1 mm^3 (see Chapter 2, Section 11) in volume before beginning the substitution process. For the dehydration process, the pieces of sample in the agar gel are transferred successively into baths of varying alcohol concentrations of 30, 50, 75, and 90% alcohol in water. Each bath lasts from 5 to 15 min, depending on the sample size and type. Dehydration is completed with three baths of 100% pure alcohol (Table 2.1).

Table 2.1 Dehydration protocol example

2/3 water + 1/3 alcohol	1/2 water + 1/2 alcohol	1/3 water + 2/3 alcohol	Pure alcohol
15 min	15 min	15 min	3 × 15 min/bath

Large samples are more difficult to dehydrate, especially if they contain molecules that adsorb water such as collagen, glycoproteins, mucus.

Dehydration will be much better if the sample is fixed properly, which requires very small samples.

Substitution

Substitution differs slightly, depending on whether an epoxy or acrylic resin is used. Both types of resin contain several components mixed at the last minute according to a given ratio and based on the hardness to be obtained and the manufacturer's instructions. In particular, the addition of the accelerator or initiator (for acrylic resins) triggers resin polymerization. The mixture containing the accelerator must be used within a short period of time, from 1 to 24 h depending on the resin and usage temperature.

The epoxy resins most commonly used contain diglycidyl ether (replacing Epon) or araldite as monomers, and MNA or DDSA as anhydrides. ERL is a low-viscosity monomer; it is used following the Spurr protocol (see "Methodology", Chapter 4). These are all good infiltration–embedding media.

The procedure for relatively viscous and non-polar epoxy resins is as follows: Substitution is carried out in intermediate baths of resin and alcohol and is finished with several baths of pure resin, 5–15 min each.

The changes occurring in the sample can be monitored visually. Resin is heavier than water; samples containing more water than the mixture float on the surface or in the middle of the liquid. As long as the sample has not yet fallen to the bottom of the solution, this step must be prolonged.

With fairly viscous epoxy resins, and in difficult cases, each bath can be doubled in time or extended up to 12 h. Baths can also be planned with constant stirring to facilitate the exchanges. If this is not enough, an intermediate solvent can be used between the alcohol and the resin, which is more miscible with the resin and reduce viscosity. This solvent is 1,2-epoxypropane (EP). In this case, three baths containing alcohol and progressively more 1,2-EP content are prepared, one or two baths of pure 1,2-EP, and three baths of the resin + 1,2-EP mixture, finally ending with pure resin (Table 2.2).

Table 2.2 As an example of a possible substitution procedure to follow, the protocol below contains six steps, from pure alcohol to pure resin

2/3 alcohol + 1/3 EP	1/2 alcohol + 1/2 EP	1/3 alcohol + 2/3 EP	Pure EP
15 min	15 min	15 min	2 × 15 min baths
2/3 EP + 1/3 resin	2/3 EP + 1/3 resin	2/3 EP + 1/3 resin	Pure resin
15 min	15 min	15 min	1 h

The sample is left overnight in the pure resin mixture. Embedding is performed the following morning. The sample is then transferred into a special mold filled with resin and left to polymerize (see Chapter 2, Section 8 on Embedding). This procedure can be modified depending on the sample type.

Acrylic resins are usually polar and much more fluid before polymerization than epoxy resins. They contain many mixtures of polymers that are often not identified in commercially available products. They generally polymerize at 333 K, often with a risk of increased volume and irregular polymerization inside the sample. This is why polymerization is preferably carried out at 277 K under UV light. These resins are often more difficult to cut via ultramicrotomy (see Chapter 4, Section 4). Their polar function can be used to successfully produce many cytochemical reactions on the thin section. These resins are not very stable under the electron beam and they often polymerize unevenly in the presence of osmium. Osmium concentrates heat (during polymerization at 333 K) or light (during polymerization with UV light), creating harder cores and out-gassing within the sample.

There are different commercially available mixtures (LR white, Unicryl, and Lowicryl) that are likely to penetrate and polymerize the sample below 273 K.

Since these resins are polar, the final dehydration steps can be performed directly in pure resin. The technique also allows for infiltration before embedding, following the same procedure as with epoxy resins.

9.3 Variants

9.3.1 Variant 1

For porous samples, the air is replaced under a vacuum with a liquid (water or alcohol) and then the technique is carried out as in the general case.

9.3.2 Variant 2

In the case of single-layer cells from culture, embedding will directly follow dehydration without performing the infiltration step. Capsules filled with resin are overturned directly on the cell layer.

9.3.3 Variant 3

If an immunological staining has to be performed on an ultramicrotome section in order to better preserve the antigen sites, dehydration is performed at decreasing temperatures down to 253 K (Lowicryl K4M or LR white). Infiltration and embedding will be performed at the same temperature. This technique is called "progressive low temperature" or PLT and requires special equipment to regulate the temperature.

Protocol Example

- Chemical fixation for 15 min at 293 K in a paraformaldehyde 1 + 0.25% buffered glutaraldehyde fixative mixture
- Rinsing with a suitable buffer (three quick baths)
- 30% alcohol in distilled water at 293 K for 10 min
- 50% alcohol in distilled water at 273 K for 10 min in the special enclosure
- 75% alcohol in distilled water in the special enclosure with temperature decreases down to 263 K for 30 min
- 80% alcohol in distilled water in the special enclosure with temperature decreases down to 253 K for 30 min
- 90% alcohol in distilled water in the special enclosure with temperature at 253 K for 30 min (up to 1 h)
- 100% alcohol at 253 K for 30 min, two times
- Infiltration in a mixture of 1/3 Lowicryl K4M resin + 2/3 100% alcohol at 253 K for 1 h
- Infiltration in a mixture of 1/2 Lowicryl K4M resin + 1/2 100% alcohol at 253 K for 1 h

- Infiltration in a mixture of 2/3 Lowicryl K4M resin + 1/3 100% alcohol at 253 K for 1 h
- Finally, two baths in the pure resin mixture, then embedding
- Polymerization under UV light (wavelength > 360 nm) for 24 h at 253 K followed by 48 h at 293 K

9.3.4 Variant 4

Fine particles are concentrated at the bottom of a conical plastic capsule. Dehydration and embedding will be performed directly in the capsule, proceeding with a re-suspension followed by centrifuging at each step.

Agar embedding (after chemical fixation) can also be carried out. The small agar block will then be handled like a normal block (Fig. 2.40 shows an example of this procedure).

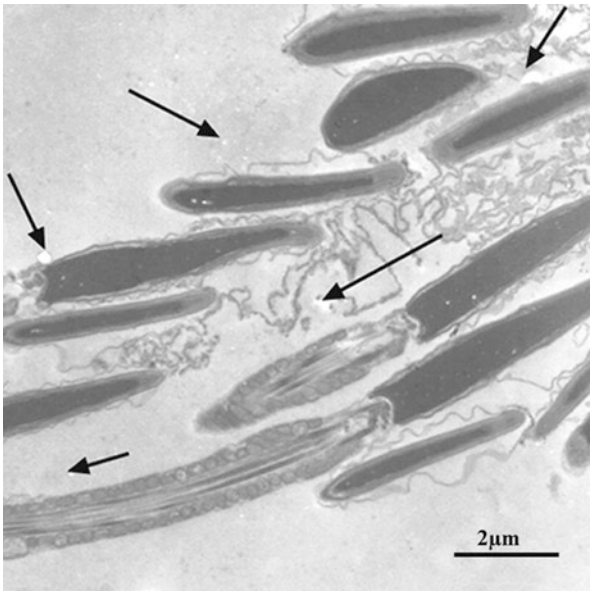

Fig. 2.40 Dog spermatozoa chemically fixed, agar infiltrated, and then resin embedded. Poor agar dehydration leaves small holes (*arrows*) in the epoxy resin (*J. Boumendil, CMEABG, UCB-Lyon 1*)

9.4 Advantages

This preliminary preparation technique is used to homogenously harden a sample, to fill in the gaps of porous material and to avoid shape modification while ultramicrotomy sectioning. Epoxy resins are perfect for ultrastructural investigations.

Acrylic resins and the Spurr mixture are very useful for difficult-to-embed samples (dermis, bone, plants, etc.).

Polar acrylic resins are recommended for carrying out immunological staining or other sample labeling.

Non-polar acrylic resins are recommended for chemical analysis.

9.5 Limitations

This preliminary preparation involves organic solvents that can dissolve a sample's compounds (lipids and triglycerides) and cause the displacement or loss of ions and small molecules dissolved in water. This preparation creates a very fine granular morphology in the TEM sample preparation that limits the image resolution.

In some cases, embedding may not be satisfactory when

– it contains holes due to the presence of air microbubbles that were not eliminated (Fig. 2.40). This artifact is common when performing agar embedding before dehydration: Either the agar contains air microbubbles or the agar dehydrates poorly
– in the case of bacteria such as the staphylococci in Fig. 2.41, the presence of a glycoprotein wall impedes dehydration and embedding

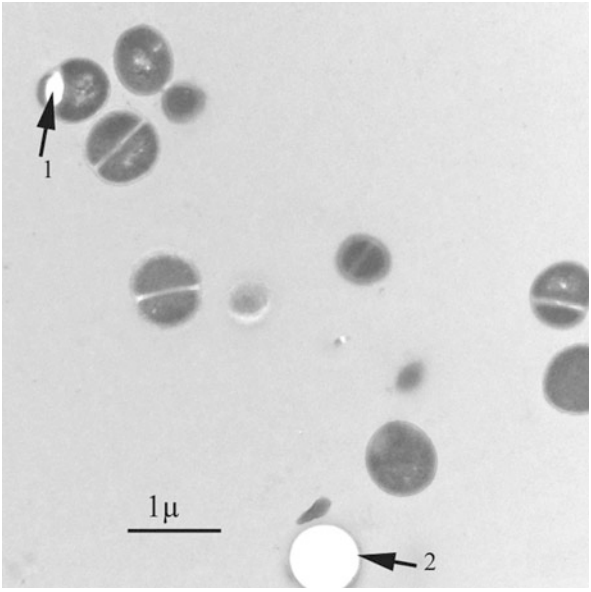

Fig. 2.41 *Staphylococcus aureus* chemically fixed, agar infiltrated, and then resin embedded. One bacterium is poorly infiltrated (1) and another one has disappeared (2) (*A. Rivoire, Ezus, UCB-Lyon 1*)

– hardness inhomogeneity will make sectioning difficult or impossible. This drawback is due to poor resin penetration and poor water elimination. If the water is not fully removed, this means that dehydration and possibly fixation were not sufficient. The technique must be started over, increasing the time in each bath, doubling the baths, and stirring the sample to improve the exchanges. If possible, a more fluid resin also should be used

Inhomogenous embedding will result in different defects in the section, such as

– folded slice means the block was too soft;
– a slice presenting colored bands means the block was not of a homogeneous hardness;
– a slice that tears at the junction between the tissue and the resin means too great a difference in hardness between the resin itself and the poorly embedded material, corresponding to a poor choice of resin and
– a slice that breaks up on the water surface means tissue presenting unembedded areas that do not resist the water's surface tension.

9.6 Compatible Techniques

Ultramicrotomy, cryo-ultramicrotomy, FIB, positive-staining contrast, and immunostaining.

9.7 Risks

These are related to carcinogenic chemicals (epoxy or acrylic resins) or organic solvents (1,2-epoxypropane, etc.). Caution must be taken as the vapors from acrylic resins are very toxic, as is contact with the skin. The use of gloves during the procedure is strongly recommended.

Dehydration–infiltration–embedding should always be performed under a fume hood.

9.8 Conclusion

Paired with chemical fixation, this is a base preliminary preparation for biological samples (Fig. 2.42). It is also used to prepare fine particles containing a liquid solution for ultramicrotomy. The results should be compared with other cryogenic techniques: substitution–infiltration–embedding in cryogenic mode, cryo-ultramicrotomy, frozen hydrated films of single particles, and freeze fracture.

Removal
Aldehyde fixation in a final 1-2% concentration in an osmolarity and pH adjusted buffer 1-24 hours at room temperature
Rinses in the osmolarity and pH-adjusted buffer 3 successive rinses for 10 minutes at 277 K These rinses can last up to 24 hours at 277 K
Post-fixation in osmium tetroxide in a final concentration of 0.5-2% in an osmotically and ph-adjusted buffer. 1-2 hours maximum at room temperature If the time is greater, the concentration is 0.5% and temperature is 277 K
Quick rinse in distilled water
Dehydration in a series of alcohol baths from 30% to 100% (ten minutes each)
Infiltration: *Optional intermediate step: 2 baths in 1.2 epoxy propane in increasing concentrations diluted in alcohol. Each bath is for 10 minutes at room temperature* 1st bath for 10 minutes at room temperature: embedding resin in a concentration of 1/3 100% alcohol or in 1.2 epoxy propane. 2nd bath for 10 minutes at room temperature: embedding resin in a concentration of 1/2 100% alcohol or in 1.2 epoxy propane. 3rd bath for 10 minutes at room temperature: embedding resin in a concentration of 2/3 100% alcohol or in 1.2 epoxy propane. 4th bath for 1 hour or overnight at room temperature in pure resin
Embedding: in a mold or capsule in pure resin **Polymerization** in a sterilizer at 333 K for 3 days on average.
Slices
Contrast 1: Uranyl acetate at 4% diluted in water or alcohol from 10 minutes to 1 hour at room temperature or at 333 K. Allow to dry. **Contrast 2**: Lead citrate diluted in a buffer, with a pH of 10, for 4 minutes at 293 K
Observation

Fig. 2.42 Protocol for all of the chemical techniques

10 Substitution–Infiltration–Embedding at Low Temperatures

10.1 Principle

This preliminary preparation technique results in a hard, workable block of material containing a liquid phase, which then can be used to produce a thin slice by ultramicrotomy. Cold physical techniques are used in order to prevent artifacts that may be associated with classical chemical techniques in particular. After solidification of the material's liquid phase through freezing, the technique consists of substituting

this solid phase with an organic solvent (alcohol or acetone) at low temperature. Infiltration and embedding in a resin are usually performed at a low temperature by a method known as cryo-substitution. Another method consists of sublimating the ice under a vacuum, called cryo-sublimation or freeze drying.

The technique described in this section can be applied to biological materials, polymers, or minerals, as well as to bulk materials or fine particles in liquid solution.

10.2 Operating Mode

10.2.1 Equipment and Supplies

For cryo-substitution the equipment consists of a liquid nitrogen-cooled chamber with temperature settings from 153 to 277 K, a stirrer, a UV lamp, a vacuum chamber, a centrifuge, a stirrer, and special molds. An electronic box is used to control the temperature, time, and to schedule temperature increases.

For cryo-sublimation the equipment also includes a pumping system (using absorbtion pump) to make a vacuum in the chamber.

10.2.2 Procedure

After fixing the sample using the physical technique of freezing, this technique contains three steps, which are all carried out at a low temperature:

1. Cryo-substitution: replacing the water in solid phase with an organic solvent
2. Cryo-infiltration: substituting the solvent with an acrylic resin such as Lowicryl
3. Cryo-embedding: embedding the sample in a resin such as Lowicryl and polymerizing with UV radiation at low temperatures

Cryo-substitution

The chamber is filled with nitrogen and the temperature is set to 183 K. Small containers filled with solvent (ethanol, methanol, or acetone) are placed in the chamber. When the temperature is steady at 183 K, the sample is frozen and transferred from the liquid nitrogen to one of the containers holding the solvent. The sample is left for 24 h at 183 K, and then a temperature increase of 5 K/h is scheduled until 223 K is reached. This temperature is maintained for 24–48 h, changing the solvent bath every 24 h. Depending on the type of resin, sublimation in the resin can occur at this temperature or we can schedule another temperature increase (see Protocol a, Table 2.4). The resins used are acrylic resins capable of polymerizing at low temperature. They are marketed under the brand name Lowicryl and are made of mixtures of several monomers. There are four types of Lowicryl: two are non-polar, HM20 and HM 23, and two are polar, K4M and K11M. These resins polymerize under ultraviolet light in the presence of an initiator (benzoyl peroxide). Table 2.3 summarizes the polymerization temperature limits for each resin.

Table 2.3 Different types of Lowicryl resin

Lowicryl	HM23	HM20	K11M	K4M
	Non-polar	Non-polar	Polar	Polar
Polymerization temperature limit	213 or 203 K	223 K	223 K	253 K

Cryo-infiltration

Cryo-infiltration is carried out in intermediate baths of resin and solvent and finished with several baths of pure resin. The exchanges lessen as the temperature lowers; therefore, the bath times will have to be longer and more numerous.

Embedding

The sample is transferred to a special mold filled with resin while remaining in the cooled chamber. A UV lamp is placed over the chamber, which enables the polymerization, while maintaining the temperature at the desired level (between 213 and 253 K, depending on the situation). Polymerization time at this temperature is from 24 to 48 h and will be followed by a temperature increase to 277 K, where polymerization will be completed in less than 24 h.

It takes 1 or 2 weeks to perform the entire technique (see Protocol a, Table 2.4).

10.3 Variants

10.3.1 Variant 1

A fixation procedure can be carried out during cryo-substitution by adding paraformaldehyde, glutaraldehyde, or even osmium tetroxide at a concentration of 0.1–1% dissolved directly in the solvent.

10.3.2 Variant 2

For samples containing air (such as plant leaves), it is necessary to perform cryo-substitution under vacuum in order to extract the air, which requires equipment that has a secondary pumping system on the chamber. In this same type of equipment we can also perform cryo-sublimation, i.e., extraction of the ice using a vacuum. Here again, we begin the technique at 183 K with steps and temperature increases throughout the procedure, ending with embedding, preferably in a non-polar resin. The technique is more difficult to carry out than cryo-substitution. It requires working on samples smaller than 0.5 mm^3.

10.3.3 Variant 3

When a large portion of dehydration has been carried out in cold (down to 253 K), the sample temperature is abruptly raised to 277 K in a final bath of 100% alcohol

and the sample is embedded in LR white resin, for example, polymerized under UV at 277 K. The advantage of this method is that it ensures better embedding than when carried out below 273 K, while protecting most dehydration-sensitive antigens.

10.3.4 Variant 4

A sample chemically fixed at room temperature may be cold-embedded to best protect the antigenicity of the proteins and in order to carry out immunolabeling. In order to do this, the sample is dehydrated in progressively colder alcohol baths after aldehyde fixation. Starting with 70% alcohol in water at 273 K, the bath is changed to 80% alcohol at 263 K and then 95 and 100% alcohol at 253 K. Embedding is performed using LR white or Lowicryl 4KM at 253 K (see Protocol b in Table 2.4).

10.4 Advantages

This preliminary preparation is used to keep the sample in a state as close as possible to the hydrated state, avoiding displacement of phases or small molecules with a minimum of substance loss and no changes in the 3D conformation of the proteins. After polymerization, the sample is brought back to room temperature and can be cut using the ultramicrotomy technique at room temperature, as with any other sample. If necessary, we can carry out positive-contrast staining using 0.5% aqueous uranyl and lead citrate. Figure 2.43 illustrates this technique. This method is particularly useful for carrying out immunolabeling on very labile antigens situated in the extracellular matrix or fixed on plasma membranes. In this case, it is preferable to use polar Lowicryl resins (K4M and K11M). Cryo-sublimation is used to remove the air from a sample, and therefore it is particularly effective in preparing aerial plant organs (e.g., leaves). It also helps to conserve ions and small diffusible molecules since we do not return to a hydrated phase. Therefore, cryo-sublimation is mostly used for chemical analyses. For this type of analysis, embedding in non-polar resins (HM20 and HM24) is preferable.

10.5 Limitations

This preliminary preparation technique requires special equipment. It is very delicate to perform and can only be done on very small samples. The block obtained following polymerization in Lowicryl resins is sometimes too soft and difficult to cut. We can improve cutting conditions by working with cryo-ultramicrotomy if polymerization is not perfect, since embedding hardness is improved by the cold.

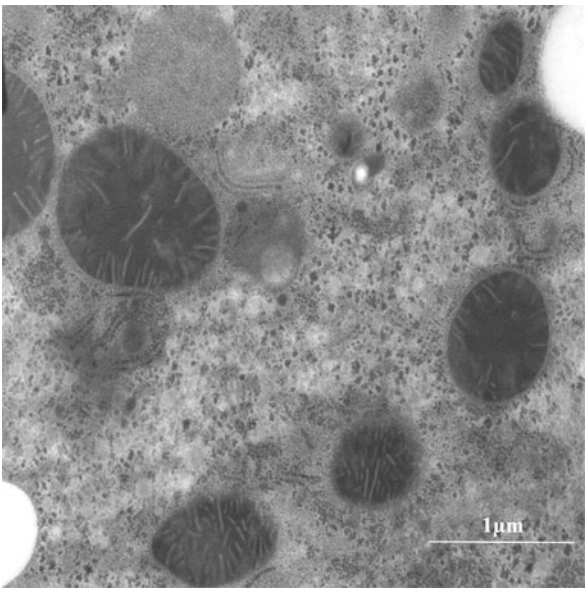

Fig. 2.43 Liver cell, cryo-fixed by ultra-rapid freezing under high pressure, cryo-substituted in ethanol + 0.5% osmium, cryo-embedded in Lowicryl K4M, cut at room temperature, and stained using 0.5% uranyl acetate in water and lead citrate. Observation in TEM bright-field mode (*J. Boumendil, CMEABG, UCB-Lyon 1*)

10.6 Compatible Techniques

Ultramicrotomy, cryo-ultramicrotomy, positive-staining contrast, and immunolabeling.

10.7 Risks

Risks are tied to the use of highly carcinogenic and allergenic embedding products (Lowicryl resins) and to the use of liquid nitrogen. This technique requires working while wearing a mask, glasses, and two pairs of gloves.

10.8 Conclusion

This is one of the most suitable techniques for EDX chemical analysis and for immunolabeling. However, the complexity, implementation time, and the often random quality of the blocks still prevent it from becoming a standard technique.

Appendix: Physical Preparation Protocols

Table 2.4 Physical preparation technique protocols

Protocol a
Ultra-rapid freezing in propane cooled with liquid nitrogen or on a nitrogen-cooled copper plate or by high pressure
Cryo-substitution and cryo-infiltration from 183 to 253 K (or 233 or 213 K depending on the situation), for several days
Polymerization at 253 K (or 233 or 213 K depending on the resin) under UV light for several days
Polymerization completed under UV at 277 K for 24 h
Ultramicrotomy with slices floating on water
Normal observation
Protocol b: progressive low temperature (PLT)
Aldehyde prefixing, 0.2% glutaraldehyde + 1% paraformaldehyde 15 min at 277 K, diluted in an osmolarity- and pH-balanced buffer
Dehydration in ethanol in several baths of increasing concentration and decreasing temperature, from 293 to 253 K (or 233 K) over several hours
Cryo-embedding in Lowicryl at 253 K (or 233 K)
Polymerization under UV in the cold (253 or 233 K), for several days. Polymerization completed under UV at 277 K for 24 h
Ultramicrotomy
Normal observation

11 Chemical Fixation

11.1 Principle

This technique consists of stabilizing strongly hydrated biological structures by creating new chemical bonds, which render the proteins insoluble. Chemical fixation is based on the bridging of proteins, transforming the protein gel into a cross-linked network. This operation enables performing subsequent dehydration while keeping structural disturbance to a minimum. It consists of creating long insoluble chains by bridging organic molecules using a chemical agent called a "fixative." This is a chemical reaction through an addition reaction at the double bonds or alcohol, aldehyde, or hydroxyl endings of the organic molecules.

This technique can be applied to strongly hydrated biological materials, bulk materials, or fine particles.

11.2 Operating Mode

11.2.1 Equipment and Supplies

This technique does not require any equipment, just laboratory glassware, pill box, micropipette, beaker, etc.

11.2.2 Procedure

The fixative solution must be prepared extemporaneously, using a fixative in a concentration of 0.5–4% placed in a vector liquid adapted to the sample's pH and molar concentration. A buffer system is used to adjust pH; the addition of a salt or sugar can be used to adjust the molar and ionic concentration. The buffer system mixture plus the salt or sugar constitutes the vector liquid.

The fixative used is one of two types: either an aldehyde (paraformaldehyde and glutaraldehyde) or a powerful oxidant such as osmium tetroxide. The technique usually includes a double fixation: the first fixation with the aldehyde and the second with the oxidant. The first acts as a cross-linking agent and the second fixes the membranes and results in the loss of their semi-permeability. Osmium tetroxide also adds contrast because of osmium's high atomic number (see "Methodology", Chapter 4).

Glutaraldehyde and osmium tetroxide fixatives penetrate the inside of tissues more or less slowly. Therefore it is essential to perform fixation on very small samples (approximately 1 mm^3).

Larger samples can be fixed using paraformaldehyde, which quickly penetrates and reacts with structures.

In all cases, once the sample stiffens, it is possible to cut it to the recommended 1 mm^3 size.

The sample must be immersed in the fixative mixture as quickly as possible after sampling.

Fixation Conditions

The fixation temperature depends on the sample size and the fixing time. If the sample is initially 1 mm^3, it can be fixed at room temperature for 1 h. If the sample is larger, it must be fixed longer, and fixing will be done at low temperatures in order to limit the risk of autolysis, because cellular enzymatic systems are less active at low temperatures. The concern with cold fixation is the slowed penetration of the fixative, and more importantly, the slowed reaction time with the tissue components. What is obtained on one side is lost on the other.

Each organism and each tissue presents itself as a particular case for which a protocol must be chosen and adapted. All of the techniques to follow fixation – dehydration, embedding, immunolabeling, freezing, or other procedures – are factors that must be taken into account when selecting the fixing mixture. Therefore, there are a multitude of protocols.

As a general rule, if used alone, glutaraldehyde is placed in a final solution of 1–2.5%. Paraformaldehyde is used in a final concentration between 1 and 4%. When the two are mixed, the final concentration is between 1 and 2% for glutaraldehyde and 0.5% for paraformaldehyde. Osmium tetroxide is used at a concentration between 0.5 and 4%.

Below is an example of a classical chemical fixation protocol for a hydrated bulk biological material applied to the study of mammal ultrastructures:

– Fixation for 1 h at room temperature in the following fixative mixture: Glutaraldehyde in a 2% final concentration and paraformaldehyde in a 0.5% final concentration diluted in a phosphate buffer in a final concentration of 0.1 M (mono-Na/di-K) at a pH of 7.3.
– Washing in five baths for a period of 10 min each (at room temperature or 277 K) in a vector liquid containing a final concentration of the phosphate buffer of 0.175 M (mono/di-K) at a pH of 7.3.
– Osmic post-fixing for 1 h at room temperature in the following mixture: osmium tetroxide in a 1% final concentration in a 0.1 M phosphate buffer (mono-Na/di-K) at a pH of 7.3.
– Very quick rinse in distilled water before beginning dehydration.

Living samples must be removed as quickly as possible and immersed in the fixative solution. The sample is immediately cut into small pieces between 1 and 2 mm across, either into a cube shape if the preparation orientation is random or in a parallelepiped shape if a longitudinal or transverse orientation is required. The sample is left at room temperature for 1–24 h in this mixture, and then rinsed several times in the vector liquid, always respecting the pH and molar concentration. The sample is placed in the second fixative mixture for 1–24 h, and then quickly rinsed in distilled water before being dehydrated in a series of alcohol baths of increasing concentration up to pure alcohol.

After impregnation and embedding (see Chapter 2, Section 9) are performed in order to be able to perform ultramicrotomy (see Chapter 4, Section 4), positive-staining contrast (see Chapter 7, Section 3) and TEM bright-field observation follow. Figure 2.44 shows an example of tissue prepared following this set of techniques.

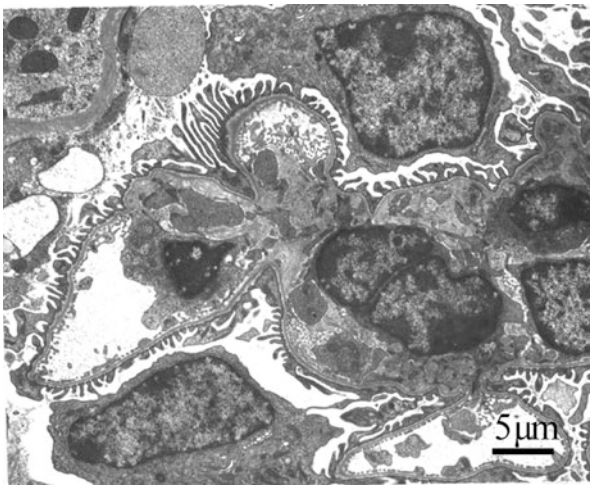

Fig. 2.44 Renal glomerulus obtained by double fixation in glutaraldehyde/osmium, dehydration, embedding in epoxy resin, ultramicrotomy and positive-staining contrast with uranyl and lead (J. Boumendil, CMEABG, UCB-Lyon 1)

Different protocols are possible depending on the type of sample and subsequent observations; in order to find out more, see Chemical Protocol in Table 2.2, for the succession of operations.

Comment: The sample can be stored at 277 K in the final vector liquid rinse, either overnight or for several days, e.g., while awaiting other samples.

It is not common to observe images from a sample fixed only with glutaraldehyde, because there is no contrast.

11.3 Variants

11.3.1 Fixation for the Immunolabeling and Cytochemical Techniques

In the immunolabeling technique, the fixative mixture contains 1% paraformaldehyde with between 0.1 and 0.5% glutaraldehyde. Samples will be fixed for a very short time, from 10 to 30 min, and the rinses will be quick.

After a rinse in the buffer, the immunological reaction is carried out, followed by osmium tetroxide post-fixation.

Likewise, in certain cases with cytochemical labeling, the reaction occurs after aldehyde fixation, but post-fixation can be carried out before embedding. Figure 2.45 shows this type of sample in which alkaline phosphatase in the lysosomes has been highlighted by a chemical reaction coupled with a lead salt that

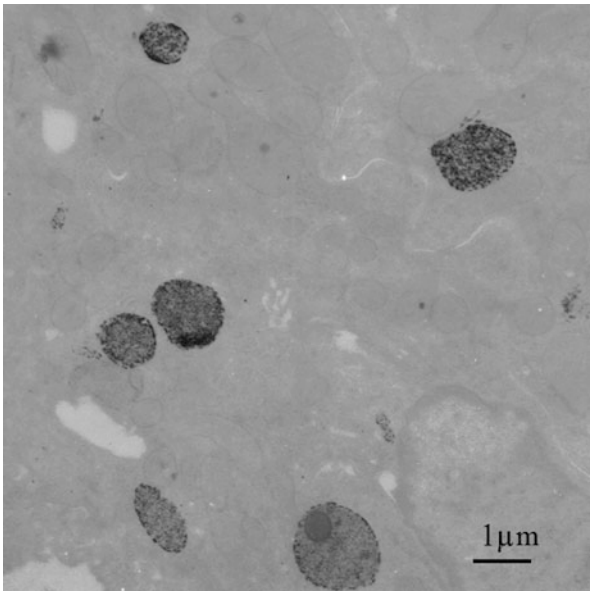

Fig. 2.45 Cell from the proximal renal tubule prepared with double glutaraldehyde fixation, osmium embedding, and ultramicrotomy. Lysosomes are labeled by means of a cytochemical reaction (*J. Boumendil, CMEABG, UCB-Lyon 1*)

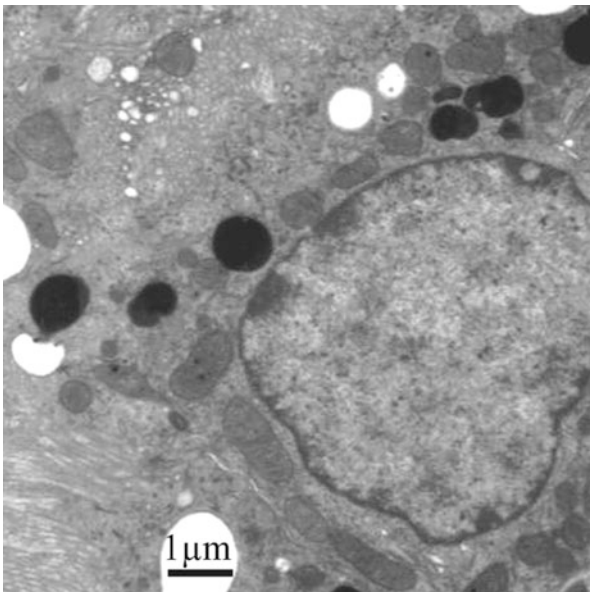

Fig. 2.46 Same type of cell with additional positive staining. Lysosomes are black after positive staining (*J. Boumendil, CMEABG, UCB-Lyon 1*)

makes the labeling observable. Observation is without positive contrast. Figure 2.46 represents a classic full preparation, double fixation, embedding, cutting, and positive staining of the same type of cell.

11.3.2 Fixation of Materials Containing Air

In the case of samples containing air (lung, plant leaves, etc.) the sample is immersed in the fixative mixture and the container is directly connected to a pumping system (a rotary pump or, even better, a liquid jet vacuum pump), to degas the sample while making sure not to draw it up into the pump. Air bubbles are seen rising to the surface. This pumping is sufficient when, after it is discontinued, the sample sinks and is drawn back to the bottom of the liquid. After placing the sample in a newly prepared fixative mixture, we proceed with the technique as in a general case (Fig. 2.47).

11.3.3 Fixation of Materials by Perfusion

In the case of organ fixation in vertebrates, and more particularly mammals, where it is difficult to have access for sampling, e.g., the nervous system, a perfusion fixation protocol is used.

This perfusion is performed by injecting the fixative into the bloodstream to replace the blood flow while respecting osmotic and ionic balance, as well as pres-

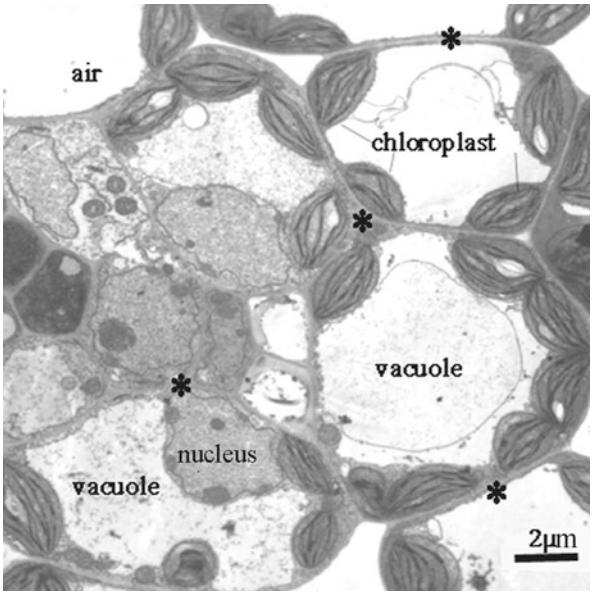

Fig. 2.47 Duckweed leaf. Plant cells double fixated in McIlvain buffer. Cell walls (*asterisks*) and large liquid and air vacuoles can be seen. The air was replaced with an embedding resin according to the technique described in the variant (*R. Pepin and J. Boumendil, CMEABG, UCB-Lyon 1*)

sure and flow rate. This technique is used to prevent anoxia in tissues that would have required too long a removal time before immersion into the fixative mixture (Fig. 2.48).

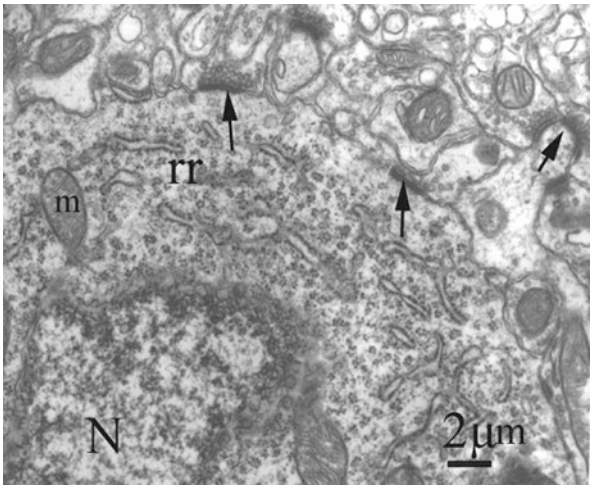

Fig. 2.48 Rat central nervous system obtained using fixation with glutaraldehyde perfusion, osmic post-fixation, embedding in epoxy resin, ultramicrotomy, and positive-staining contrast with uranyl and lead. N: nucleus, m: mitochondria, r r: rough endoplasmic reticulum, *arrows*: synaptic endings (*J. Boumendil, CMEABG, UCB-Lyon 1*)

11.4 Advantages

This technique is used to perform dehydration using alcohol or acetone without destroying the overall organization through water loss. Furthermore, alcohol has a harmful effect on living proteins because it coagulates and denatures them quickly. Once fixed, the proteins no longer undergo this type of deformation.

This technique is used to make structural observations that do not exactly represent reality, but by comparing different samples processed in the same way it can be used to determine functional differences (normal or pathological state, presence of infectious agents, synthetic activity within cells, etc.).

By fixing heavy osmium atoms, the preparation shows a useful electron contrast.

11.5 Limitations

This technique changes the structure and texture of living organic matter. It changes the distribution of molecules within the structure, binding them with molecules that might have been independent. It constitutes a non-negligible addition of chemical molecules. It leads to a loss of elements from the sample: diffusible ions, very small molecules (highly soluble in water or alcohols) such as sugars or lipids, which do not react with fixatives (Fig. 2.49).

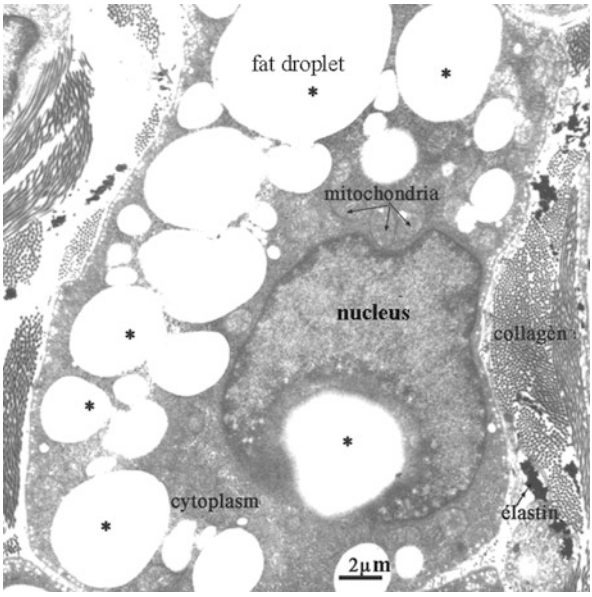

Fig. 2.49 Adipocyte. Double fixation in a phosphate buffer, saturated lipids are not fixed. Instead there are lipid pools (*asterisks*) that the embedding resin has filled and which are transparent to electrons (*J. Boumendil, CMEABG, UCB-Lyon 1*)

The chemical fixation technique leads to the creation of many artifacts. The main artifact results from the technique itself. Indeed, fixed biological material is no longer the same as the natural material. Only the observation of samples processed in the same way enables the samples to be compared; however, we cannot in any case use this technique to observe living, hydrated organic matter. Many changes occur.

11.5.1 Spatial Conformation Changes

The proteins undergo deep changes during chemical fixation, the main one being a change in their spatial conformation. This drawback will be a major one in immunological detection because reactive antigenic sites can be masked or destroyed. It changes their ionization and increases the risk of dissolution in water, and therefore their loss during dehydration.

Since glutaraldehyde has double binding potentialities with proteins, it creates artificial bridging that can lead to interpretation errors during labeling. This is why it is preferable to use the paraformaldehyde (a monoaldehyde).

11.5.2 Loss of Chemical Elements

Chemical fixation does not conserve saturated lipids or sugars (except for complex sugars such as glycogen or starches). Free ions, or those barely bound to structures, also risk being removed or displaced, which affects the analysis of chemical composition.

11.5.3 Structural Changes

Lastly, osmium tetroxide fixation deeply modifies the structure of the phospholipid components of the membranes. It causes the transformation of membrane phospholipids, which are globules, into unbroken lines. This gives the preparation its compartmentalized aspect, separated by fixed membranes. Indeed, it bridges radical phosphates by creating a double-continuous barrier locking in the lipid poles, which leads to the canceling of its semi-permeability and makes it appear as a double line opaque to the electrons. The advantage is that after osmotic fixation, the tissue can be processed with distilled water or alcohol without being subjected to any new damage.

11.5.4 Structural Transformation: Artifacts Due to the Tissue-Sampling Method

Anoxia and autolysis, i.e., the destruction of organic matter by tissue enzymes, can occur if sampling is not quick enough after the death of the organism and the interior fluid circulation stops (blood circulation for mammals, lymph circulation for insects or crustaceans, etc.). In this case, we observe either degradation in the mitochondrial matrix or bright areas in the cytosol. If the sample is not immersed immediately

in the fixative mixture, the sample is dried out and water and ion movements are created. We can also cause tearing within the sample by trying to cut very small pieces (1 mm^3) of a soft tissue. One solution is to let a relatively large sample (1 cm^3) harden in the aldehyde fixative mixture before making small cuts or to use a vibratome, a special device intended for this type of cutting. Generally speaking, in order to prevent these sampling artifacts, it is recommended to plunge a large part of the tissue into the fixative mixture before sampling. If this is not possible, the circulating fluid should be replaced with the fixative mixture by carrying out a perfusion.

11.5.5 Chemical Changes: Artifacts Linked to the Composition of the Fixative Mixture

We have seen that artifacts can form because of pH and molar or ionic composition defects. In order to prevent these artifacts, we must know the circulating fluid that bathes the cells and try to reconstitute it in the vector liquid.

A pH defect will manifest itself by extraction of cytoplasmic proteins if the medium is too acidic and by poorer conservation of nucleic acids in a medium that is too alkaline. Variations in pH generally cause disrupted conservation of some organelles (mitochondria, reticulum, etc), as shown in Figs. 2.50 and 2.51.

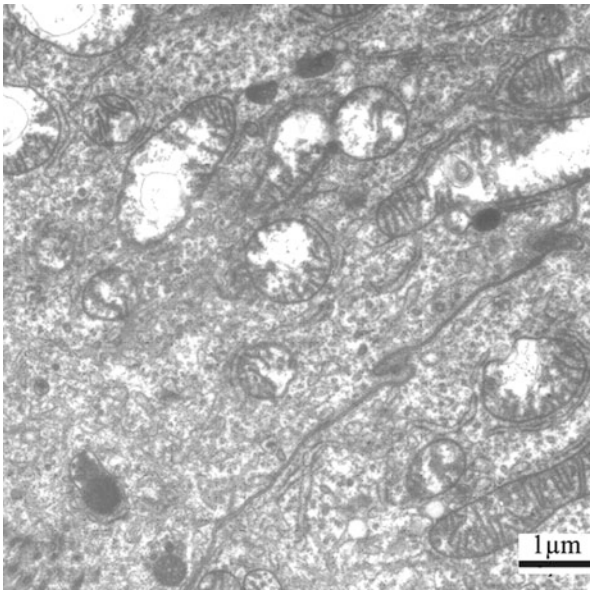

Fig. 2.50 Duodenum cells with deteriorated mitochondria. Their internal matrix is washed out and forms myelinic figures. This artifact is due to a poor pH and ionic balance (*J. Boumendil, CMEABG, UCB-Lyon 1*)

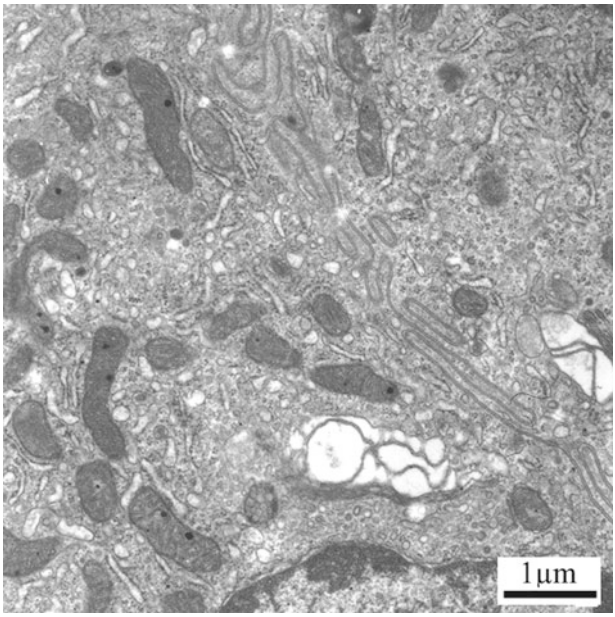

Fig. 2.51 Duodenum cells with normal mitochondria. They are not dilated in the club-shaped endings. The internal matrix is dark. In both cases, we performed double fixation, embedding, and uranyl/lead contrast (J. Boumendil, CMEABG, UCB-Lyon 1)

Water loss from cells, caused by a hyper-osmotic fixative mixture, results in structural condensation and a compact aspect. Water entry into cells, caused by a hypo-osmotic fixative mixture, results in structural swelling and a washed-out aspect. Ionic imbalance creates disturbances in cell organelles.

With experience we will see that the results also vary depending on the type of buffer. With the phosphate buffer, we observe better conservation of internal membrane systems (reticula, nuclear membrane, mitochondrial cristea) than with a cacodylate buffer. With an organic buffer, ionic neutral, there will be less water displacement between the cell and the extracellular medium, and therefore better cytosol preservation.

The presence of sugar in the vector liquid, used as an osmolarity corrector, makes the fixative liquid more viscous. It slows the fixative penetration and its reactive capacity with tissue proteins. Therefore, structure will be less well preserved. Furthermore, it is difficult to eliminate during dehydration, and we will notice a structural thickening during observation.

Finally, we may note that despite pH, molarity, and ionic concentration adjustments, there is always an increased sample volume during aldehyde fixation and a volume loss during osmotic fixation.

11.6 Compatible Techniques

FIB, ultramicrotomy, cryo-ultramicrotomy, freeze fracture, negative staining, and positive staining.

11.7 Risks

These are risks tied to the use of carcinogenic (aldehydes) or corrosive (oxidants) chemicals.

11.8 Conclusion

This is the basic preparation for biological samples. It is an essential step for dehydration and embedding in resin. These steps constitute the chemical preparations of a bulk biological material required to obtain thin slices using ultramicrotomy. For the past 10 years, we have tried to replace physical fixation (cryo-fixation, see Chapter 2, Section 12), as it is still very difficult to perform correctly on samples from 0.5 to 1 mm^3 in size. However, cryo-fixation is commonly used for very small samples in suspension (see Chapter 6, Section 2).

12 Physical Fixation: Cryo-fixation

12.1 Principle

This technique is used to stabilize hydrated biological structures simply through the vitrification of the aqueous phase. It consists of sharply and rapidly lowering the sample temperature so as to transform the water it contains into vitreous ice, enabling either direct observation in the frozen phase or subsequent dehydration. In the latter case, even if the water is subsequently removed, the structure was immobilized in its hydrated state; therefore it is much closer to the native state. This technique is based on a physical effect essentially involving temperature variations of the system. These can be combined with pressure increases. This technique can be applied to very hydrated biological materials and polymers, bulk materials, or fine particles.

12.2 Operating Mode

12.2.1 Equipment and Supplies

The technique requires equipment of varying complexities depending on whether the sample is bulk or fine particles. This may be a simple device for dipping the sample in a cooled fluid, a device for projecting the sample on a cooled

metal, or a very complex device applying high pressure (210×10^6 Pa) when cooling.

12.2.2 Procedure

In order to obtain vitreous ice from pure water, the temperature must be lowered to less than 93 K at a rate of 10^6 K/s. For a biological sample rich in solutions and proteins, vitrification can occur with a decrease of 10^3 K/s (see "Methodology", Chapter 4, Section 5).

Physical Characteristics of Freezing

The technique uses a very low-temperature freezing agent that can be a liquefied gas or a metal cooled by a liquefied gas.

The fluid with the lowest temperature, which can be used without too much risk of explosion, is liquid helium at 2 K. The easiest to obtain is liquid nitrogen at 77 K. Liquid propane or liquid ethane works as well.

The freezing agent will also be selected depending on its thermal conductivity, which is a thermodynamic constant characterizing a substance during heat transfer by means of conduction. It corresponds to the quantity of heat transferred per unit of surface area. For gases, we refer to diffusivity. The higher the diffusivity coefficient, the faster the sample will be cooled. Liquefied propane or ethane has better thermal diffusivity coefficients than liquid nitrogen.

The coefficient is less in the gaseous phase of a substance. A liquefied gas purchased commercially is balanced at its boiling point (e.g., 77 K for liquid nitrogen). The Leitendorf effect or calefaction phenomenon results from the heating of the liquefied gas by a warm object (371 K), which causes significant degassing. The specimen to be frozen is no longer in contact with the liquid at 77 K, but rather with a gaseous layer at a much higher temperature and with much lower thermal conductivity. As a result, freezing will occur under poor conditions.

Therefore it is important to choose a liquefied gas whose fusion and boiling points are very far apart. By cooling the liquefied gas, e.g., using liquid nitrogen (or helium), close to its solidification point, this calefaction phenomenon is kept to a minimum. For liquid nitrogen, its temperature can be lowered to near its solidification point by creating a 1.33 Pa depression. This produces slush nitrogen at around 63 K (Table 2.5).

Metals Have Better Thermal Conductivity than Fluids

The technique will then consist of projecting the sample onto the highly polished metal cooled by liquid nitrogen or helium (Table 2.6).

Table 2.5 Temperatures of Fusion and Vaporizing of Some Gases

Fluid	Solidification or fusion temperature (K)	Liquefaction or boiling point temperature (K)
Helium	2	4
Nitrogen	63	77
Propane	183	123
Ethane	93	185
Ethanol	156	351

Table 2.6 Thermal Conductivity of Some Materials

	Thermal conductivity (W/m K)		
Material	At 293 K	At 77 K	at 4 K
Silver	429	471	14700
Ultra-pure copper	401	570	11300
Gold	317	352	1710
Aluminum	237	410	15000
Plastic (PTFE)	2.5	2.4	0.5
Water	0.6	–	–
Air (for informational purposes)	0.0262	–	–

Freezing Conditions

Several factors promote the proper freezing of an organic sample, particularly the nature and volume of the sample to be frozen. The sample's water content plays a major role: A very hydrated sample will be harder to freeze correctly than a partially dehydrated sample or one in which the water is bound to proteins, sugars, or other components.

Several freezing techniques can be used depending on the type of observation to be performed and whether the material is bulk, hard, soft, hydrated, or in suspension (Table 2.7). If it is a bulk sample, the transformation to vitreous ice will be random because the thermal conductivity of organic matter is poor and the transfer of frigories to the sample core is very low. In order to try and improve the results of freezing, very small samples are used (e.g., 0.5 mm^3). If it is a suspension, the

Table 2.7 Different possible cryo-fixation methods depending on the sample type

Type of sample	Cryo-fixation method
Solution suspension of small molecules	Thin film by projection in cooled ethane
Viscous suspension	Immersion
Bulk sample: soft tissue, biopsy needle, etc.	Projection on Cu, high-pressure, immersion after chemical fixation and cryoprotection
Hard bulk sample	Immersion, high pressure

transformation to vitreous glass is obtained on a very thin film of liquid suspension plunged into a cryogenic fluid (ethane) cooled to 98 K (see the frozen hydrated film technique in Chapter 6, Section 2).

Direct Freezing Under Atmospheric Pressure: Immersion Freezing

This type of fixation is possible only on very small samples, grids holding protein solutions, and suspensions containing small particles such as liposomes, viruses, and small cell clusters. Immersion is performed directly in liquid nitrogen or better still in slush nitrogen to prevent calefaction (see "Methodology", Chapter 4). It can also be used for viscous suspensions of cells or tissues embedded in gelatin in order to directly perform cryo-ultramicrotomy. Usually, immersion freezing of cells is preceded by an aldehyde fixation and a cryoprotection bath.

Jet Freezing

This type of freezing is used for suspensions. The suspension is projected onto a side of a holey grid while a freezing fluid is projected onto the other side of the grid. Currently, quench-jet freezing is performed instead.

Jet Freezing in Cooled Ethane

This is performed in ethane cooled to approximately 98 K by a stream of liquid nitrogen. The sample is projected into the cooled liquid and the rapid entry of the sample into the freezing liquid improves freezing quality. This technique is widely used for frozen suspensions prepared for direct observation at low temperatures. It can also be used to freeze bulk samples usually fixed chemically, e.g., before freeze fracture.

Direct Freezing While Applying Pressure by Projection onto a Cooled Surface

The sample is projected onto a freshly polished copper surface cooled with liquid helium or liquid nitrogen. Projection improves the rapidity of contact between the sample and the freezing agent.

This is the only possible method for large samples or soft tissues. It is very well adapted to cellular suspensions but is not recommended for hard samples such as foil, bone, or cartilage. The freezing area will be limited to a few tens of microns in thickness from the surface in contact with the copper.

Direct Freezing Under High Pressure

This freezing technique, based on ultra-rapid freezing under high pressure (210×10^6 Pa), is currently the best technique for freezing a hydrated bulk sample. The frozen thickness of 200 μm should be well preserved. It is the ideal technique for fresh tissue, cellular suspensions, and cell cultures.

The technique consists of placing the bulk sample between two copper or gold cupels, surrounded by a neutral conduction fluid (hexadecene copolymer). The sandwich obtained with the two cupels is placed in a closed chamber capable of withstanding extremely high pressure. Freezing is initiated by a jet of ethanol, cooled by liquid nitrogen, which reaches the sample while 210×10^6 Pa of pressure is applied inside the chamber.

The technique is performed using a fairly complex device that requires delicate adjustments. In particular, the high-pressure input must be regulated with regard to the cooling fluid input. When the adjustments are perfect, it is possible to obtain vitrification of the water contained in the organic matter of a biological sample of 0.5–1 mm^3 (Fig. 2.52). At 210×10^6 Pa, the nucleation point of water is considerably reduced because water is 1,500 times more viscous than at atmospheric pressure, considerably reducing the crystal growth rate (see "Methodology", Chapter 4).

Freezing After Chemical Fixation

In some cases this costly, sophisticated, and nearly defect-free technique is not accessible. In these cases it is possible to minimize the damage introduced by poor-quality freezing by pre-fixing the sample.

This pre-fixation, in a low aldehyde concentration (0.5–2% paraformaldehyde in a pH-adjusted and molar concentration-adjusted buffer), will be brief (15 min). Fixation has the effect of creating bonds between proteins and stabilizing part of the water bound to them, which reduces the possibility of ice crystal growth.

A cryoprotectant such as sucrose, glucose, or trehalose can also be used, but they must be used after fixation in order to prevent dehydration of the cell. In the case of transmission electron microscopy, the cryoprotectant is supposed to enter the cell in order to increase its osmolarity and the viscosity of the whole. Under these conditions there is no ice formation, but these are no longer "natural" conditions for the sample, which has undergone chemical changes.

Despite this drawback, the technique is the only one for performing immunolabeling on frozen slices. In fact, fixation is essential to maintain the texture of the organic tissue in a slice between 50- and 100-nm thick, and especially for maintaining its integrity during successive impregnation with immunolabels in an aqueous medium, which occurs at 293 or 310 K. The ultrathin slice presents only a cell fraction, and without fixation it no longer has its natural coherence after warming up.

Practically speaking, the living sample must be taken as quickly as possible, blotted quickly, if necessary, and immediately projected into the freezing liquid where it will be kept for a few minutes. The resulting preparation will be kept cold in a moisture-free atmosphere (nitrogen or argon vapor) to prevent frost formation. The sample can be observed directly if its size allows, cut using cryo-ultramicrotomy, or freeze fractured. It can also undergo cryo-substitution or cryo-sublimation before being cold embedded.

12.3 Variants

The method to be used varies, depending on the type of sample and the technique used following cryo-fixation (Table 2.7). Therefore there are many protocols, as illustrated in Table 2.8.

Table 2.8 Different physical protocols of the most commonly used successions of operations

Protocol a
Ultra-rapid freezing in propane cooled with liquid nitrogen, or on a nitrogen-cooled copper plate, or by high pressure
Cryo-substitution and cryo-impregnation from 183 to 253 K (or 233 or 213 K depending on the situation), for several days
Polymerization at 253 K (or 233 or 213 K depending on the resin) under UV light for several days
Polymerization completed under UV at 277 K for 24 h
Ultramicrotomy with recovery of sections on water
Normal observation
Protocol b
Aldehyde fixation, 0.2% glutaraldehyde + 1% paraformaldehyde, diluted in an osmolarity- and pH-balanced buffer, 15 min at 277 K
Impregnation in a saturated solution of sucrose in a buffer
Freezing in slush nitrogen or propane cooled with liquid nitrogen
Cryo-ultramicrotomy and dry recovery of slices
Cryo-transfer or reheating of the slice
Cryo- or room-temperature TEM observation
Protocol c – PLT
Aldehyde prefixing, 0.2% glutaraldehyde + 1% paraformaldehyde, diluted in an osmolarity- and pH-balanced buffer, 15 min at 277 K
Dehydration in ethanol in several baths of increasing concentration and decreasing temperature, from 293 to 253 K (or 233 K) over several hours
Cryo-embedding in Lowicryl at 253 K (or 233 K)
Polymerization under UV in the cold (253 or 233 K), for several days Polymerization completed under UV at 277 K for 24 h
Ultramicrotomy
Normal TEM observation

12.4 Advantages

This is the only technique that enables fully stabilizing the structure in its hydrated state. If freezing is fast enough, there is no ice crystal growth to deform the structures, nor is there displacement of small soluble molecules or ions. It is used to make structural observations that precisely represent reality and to maintain the ionic composition and distribution of ions in aqueous phase (Fig. 2.52).

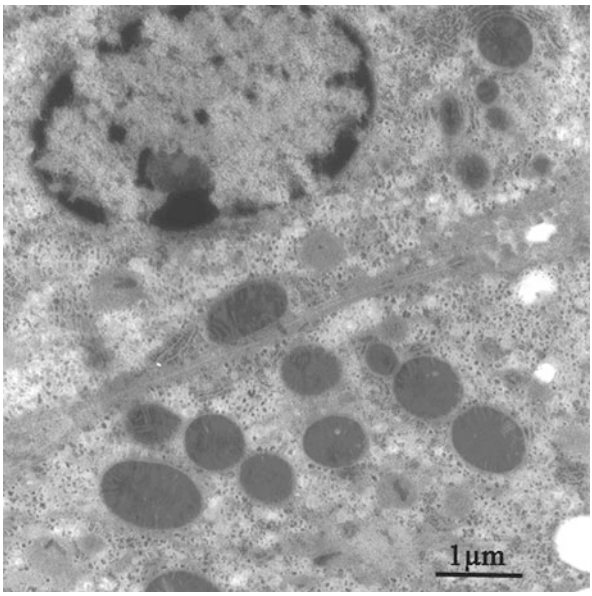

Fig. 2.52 Perfectly conserved liver cells. High-pressure freezing, freeze drying with OsO_4, cryo-embedding in K4M, cutting, uranyl/lead contrast (*J. Boumendil, CMEABG, UCB-Lyon 1*)

12.5 Limitations

This is a difficult technique, especially for bulk samples, because the act of lowering the temperature inside the sample is slowed by its volume. It also requires a very expensive high-pressure freezing device.

Vitreous ice is not stable, which means the sample should never be allowed to get warmer than 153 K or ice crystals may grow. The frozen sample risks trapping ambient moisture and must constantly be handled in a dry atmosphere. The lack of contrast makes observation very delicate and it might be useful to perform a positive staining if the analysis planned allows for it.

The main artifact results from the formation of ice crystals that appear when the temperature decrease is not fast enough. This is related to the technique employed and the sample type (bulk or suspension).

For a suspension, the film to be frozen must be extremely thin. The presence of ice crystals hinder observation, as does an overly thick ice layer, since ice very quickly becomes opaque to the electrons.

For bulk samples, the major difficulty lies in the volume to be cooled. Often the sample surface is properly vitrified, while the sample core presents crystals. The result differs depending on the technique. If the 0.5-mm^3 sample is projected into a cooled liquid (ethane or propane), there is practically always a formation of cubic crystals visible at the optical microscopic scale. Preliminary aldehyde fixation must then be carried out in order to get good freezing.

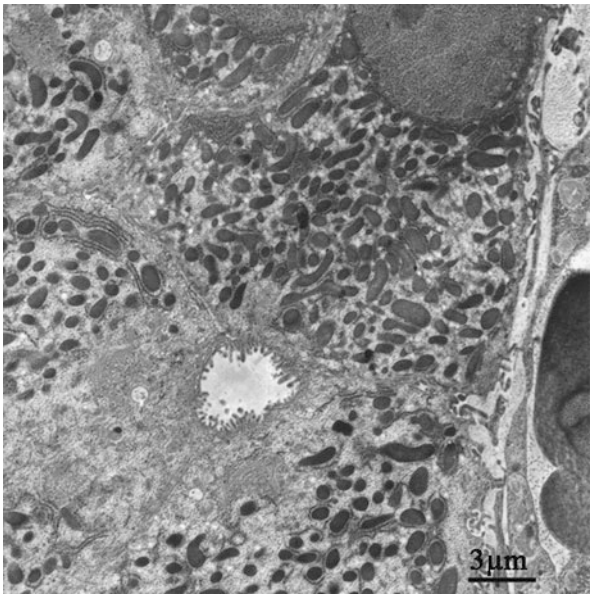

Fig. 2.53 Liver: freezing on cooled copper. The freezing front is on the right edge. Good conservation (same subsequent treatment as in Fig. 2.52) (*J. Boumendil, CMEABG, UCB-Lyon 1*)

A freezing gradient is produced if the sample is projected onto a cooled metal. In contact with the cooled metal, vitrification is correct between 0- and 10-µm thick (Fig. 2.53), then small cubic crystals are seen further in depth (Fig. 2.54), then hexagonal crystals and large poorly frozen areas (Fig. 2.55).

High-pressure freezing will help to obtain vitrification of the entire volume (Fig. 2.52), but if the equipment is not perfectly calibrated, an overpressure phenomenon in the sample or structural damage produced by the effect of the alcohol before freezing can be seen. There can also be fracturing (Fig. 2.56) within the frozen sample when the double cupels are opened.

If the temperature decrease within the sample is not fast enough, water movements produce a concentration gradient and the segregation of the "pure water" phase from the solution. Pure water freezes first, to the detriment of the solution, which gradually concentrates.

It should be noted that frozen samples present negative contrast compared to chemically fixed samples, since there is no osmium added to the membranes. Because of this, the membranes display no contrast and the cytosol is then darker than the membranes. If positive-contrast staining (uranyl or lead) is performed, the phenomenon is accentuated because the membranes do not take on the stains.

Frozen samples that do not undergo any subsequent chemical processing, and which will be cryo-observed directly, are extremely sensitive to the electron beam, which causes the diffusion of elements, a loss of matter, and local freezing of the

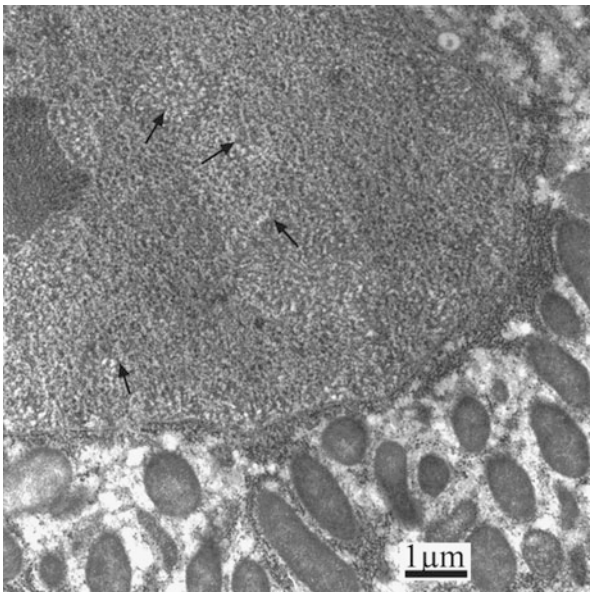

Fig. 2.54 Liver cell: freezing on cooled copper. Some tens of microns from the freezing front, very small crystals of cubic ice are appearing in the nuclei (*arrows*). Freeze drying with OsO_4, cryo-embedding in K4M, cutting, uranyl/lead contrast (*J. Boumendil, CMEABG, UCB-Lyon 1*)

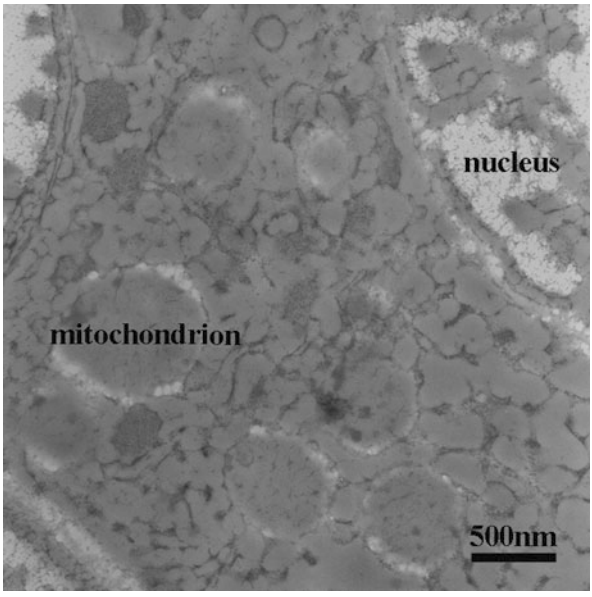

Fig. 2.55 Liver cells with many ice crystals in the barely recognizable nuclei as well as in the cytoplasm. Freezing on cooled copper, at more than 20 µm from the freezing front (same subsequent treatment as in Fig. 2.54) (*J. Boumendil, CMEABG, UCB-Lyon 1*)

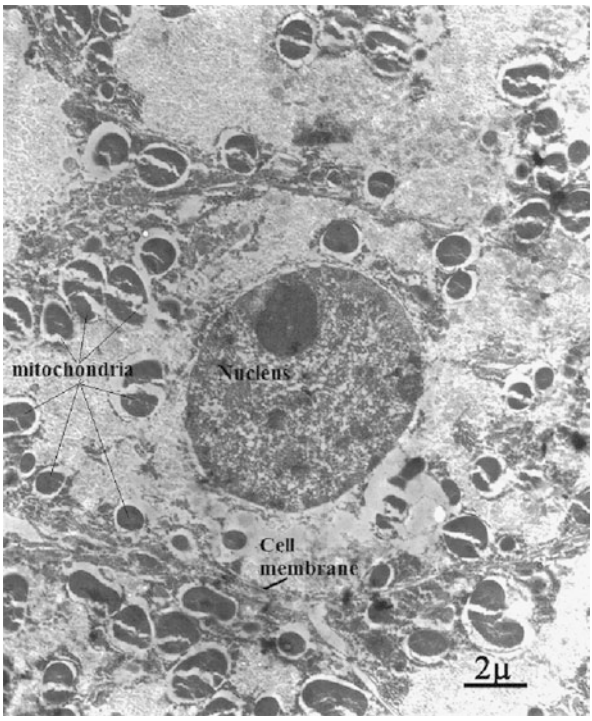

Fig. 2.56 Liver: high-pressure freezing, freeze drying, cryo-embedding in K4M, cutting, and uranyl/lead contrast. Note the absence of contrast in the membranes. All of the mitochondria are fractured (*J. Boumendil, CMEABG, UCB-Lyon 1*)

sample in the observation area. The low-dose system or a camera with a very low light level should be used.

The presence of frost on the sample is a common artifact from handling. In order to prevent frost, it is necessary to either work in a dry atmosphere (e.g., nitrogen), preventing any temperature increase above 143 K, or to operate in the presence of a dry gas. Atmospheric moisture in the microscope room can be a cause of frost formation.

Comparative investigations of freezing by projection onto a helium-cooled block and by high pressure show differences in structural preservation. In the first case, the structure is preserved but there are disturbances caused by the projection of the sample, and in the second case the molecular arrangement can be disturbed. Vitrification of water is a necessary condition, but it is not always enough to ensure proper preservation of the sample's structure.

12.6 Compatible Techniques

Cryo-FIB, ultramicrotomy, cryo-ultramicrotomy, freeze fracture, frozen hydrated suspension, positive-staining contrast, and immunolabeling.

12.7 Risks

Cryogenic burns, anoxia, and explosions.

12.8 Conclusion

This is the technique of choice for investigating the structure of very hydrated samples. It is also an indispensable technique for immunolabeling antigenic sites sensitive to fixatives or to dehydration. Only the complexity and sometimes the cost of carrying out the technique still limit its use. It is an indispensable preliminary technique for cryo-FIB, cryo-ultramicrotomy, freeze fracture, and observing films of frozen suspension.

13 Continuous Support Films

13.1 Principle

This preliminary preparation consists of making an ultrathin film on a support grid for small, brittle, or dispersed fine particle samples. This continuous film must satisfy four conditions:

– Electron transparency
– Withstanding the electron beam's effect (heating and irradiation)
– Mechanically withstanding the sample weight
– Not introducing artifacts in the image or diffraction pattern (amorphous film)

For this, amorphous films are made using materials composed of light elements such as carbon or organic compounds such as collodion, Formvar, or Triafol.

Continuous films are used as a support for any type of material, particularly for minerals, catalysts, polymers, and biological materials, or fine or isolated particles in liquid solution. It serves as a support for slices made using ultramicrotomy fine particle dispersion, and in some cases, for samples obtained using the FIB technique.

13.2 Operating Mode

13.2.1 Equipment and Supplies

Making polymer films requires an extractor hood and small laboratory supplies (crystallizing dish, glass slides, etc.). For carbon films, an evaporator is used under high vacuum. In all cases, 3- or 2.3-mm microscope support grids made of copper, nickel, aluminum, etc., are used. Grids ranging from 100 to 1,000 mesh can be chosen. Mesh refers to the number of holes per square inch.

13.2.2 Procedure

There are many procedures for making continuous films.

Collodion Film

Collodion film is obtained by spreading a solution of 1–3% collodion (nitrocellulose) dissolved in an organic solvent (isoamyl or butyl acetate) by the *solution drop method*. Support grids are placed on a metal sieve at the bottom of a crystallizing dish filled with distilled water. A drop of the collodion solution is placed on the surface of the distilled water in the crystallizing dish. The film forms immediately by evaporation of the highly volatile solvent. This film is removed with a pair of tweezers in order to remove any dust and impurities from the water. After the operation is repeated, the water is slowly evacuated using a valve located at the bottom of the crystallizing dish or by siphoning (a more delicate operation, creating turbulence that could create folds in the collodion film). The film settles on the support grids. The sieve can be recovered and the grids are allowed to dry just before use. The collodion film is very thin (approximately 10 nm, depending on the concentration of the solution used). It is very fragile and folds easily at the slightest movement of air. Another process consists of depositing grids on the collodion film and recovering both of them using a filter paper or a plastic film (parafilm), but this is more random given its fragility. The collodion film is unstable under the electron beam, especially at high acceleration voltages, and must be reinforced by a carbon deposit obtained by evaporation under high vacuum (see "Methodology", Chapter 4, Section 7). Next, the grids are laid on a thick layer of filter paper soaked with the original solvent (isoamyl or butyl acetate) and the collodion is dissolved by capillary action. This operation is long and delicate; using a pipette, solvent must be added periodically onto the filter paper around the support grids. It is hard to see exactly when the collodion is fully dissolved. In this way, only the carbon film is preserved (Fig. 2.57).

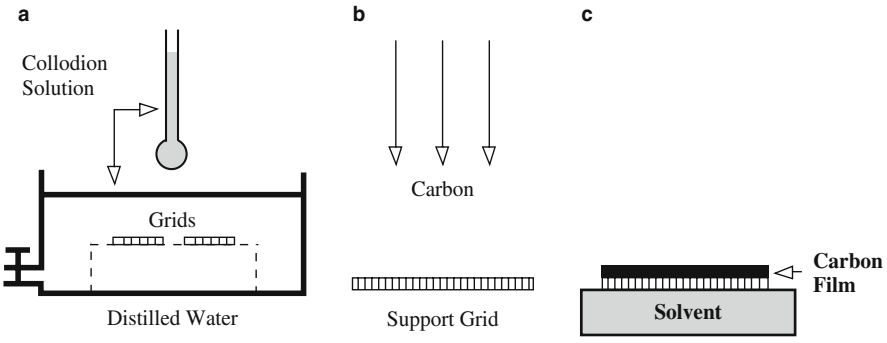

Fig. 2.57 **a** Formation of the collodion film by depositing a drop of collodion. **b** Deposition of a thin amorphous carbon film. **c** Dissolution of the collodion film

The collodion film is soluble in alcohol, ether, and most organic solvents.

The advantage of collodion film is that it can be made very quickly, but because of its fragility and because it is not stable under the electron beam, it has gradually been replaced by other types of films.

Formvar Film

Formvar (polyvinyl formal) or Triafol (butyril cellulose acetate) is obtained using the dip-float method, which consists of quickly dipping a glass slide with a good surface quality into a solution of 0.3–1% polymer in an organic solvent (1,2-dichloroethane or ethyl acetate). After the solvent evaporates, between 30 s and 2 min, the film is unstuck by slowly introducing the slide at an angle of about 30°–45° to the surface of the water in a crystallizing dish (Fig. 2.58). Unsticking occurs under the effect of the capillary force (water surface tension). In order to facilitate unsticking, we can lightly moisten the film by breathing on it and rubbing the edges of the glass slide with a razor blade. Support grids are placed directly on the floating film and are recovered by adherence onto a plastic film (parafilm) or paper. The continuous support film obtained is highly resistant both mechanically and under the electron beam and can therefore be used immediately. It is often a bit thicker than the collodion film (around 10–20 nm) and more opaque to electrons and may present a slight texture at high magnifications. In practice, it can be reinforced and made conductive through a carbon deposit obtained by evaporation under high vacuum (see "Methodology", Chapter 4, Section 7).

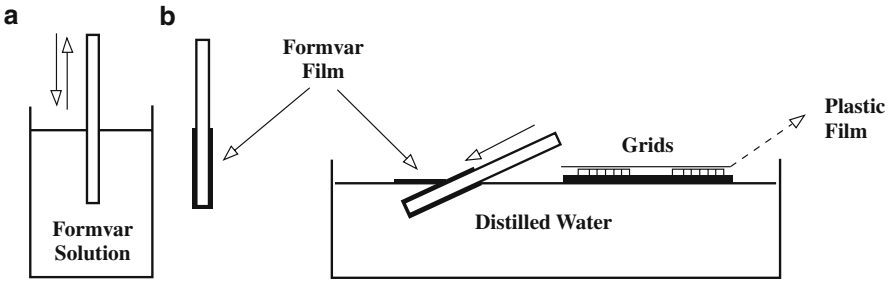

Fig. 2.58 **a** A glass slide is dipped vertically into the Formvar solution and then removed. **b** The film formed on the slide is unstuck little by little from the surface of the glass as it is introduced into the water. Support grids are placed on the film and removed by adherence onto a plastic film

Formvar or Triafol films are sensitive to solvents such as acetone and become unstable at high temperatures.

Carbon Film

Deposition onto a slide: The carbon film is obtained through sputter deposition or evaporation under high vacuum (see "Methodology", Chapter 4, Section 7). The

amorphous carbon film is evaporated onto a surface of freshly cleaved mica or onto a clean glass slide with a very good surface quality. Gem salt crystals, which cleave easily and present very flat and homogeneous surface areas of a few square centimeters, can also be used. Once made, the film is pre-cut on its support using an edge or scalpel in order to form 3-mm-sided squares; the fragments are unstuck on the surface of the water as in the dip-float method and collected one by one onto support grids that can be used immediately after drying (Fig. 2.59).

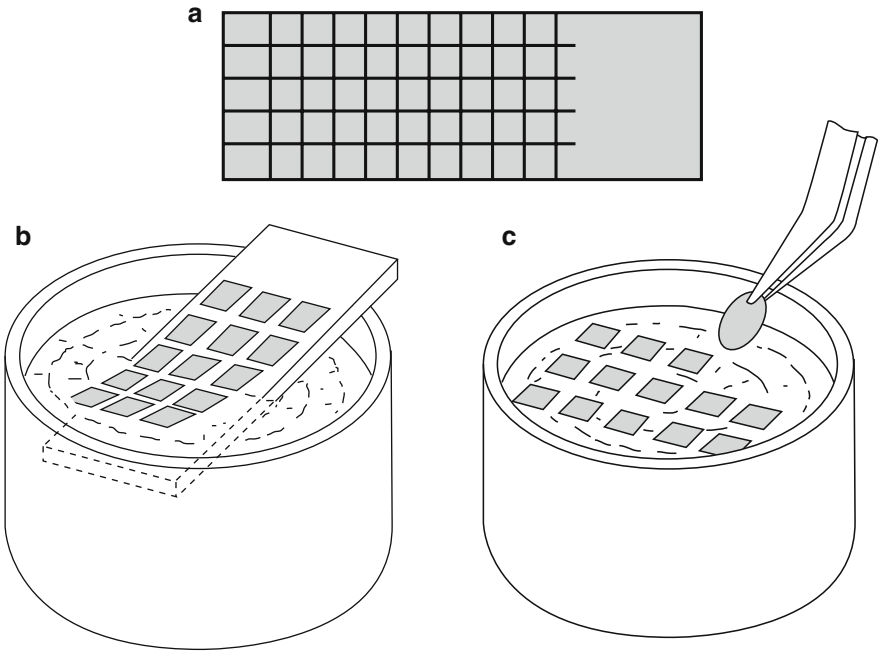

Fig. 2.59 **a** Squaring the carbon film. **b** Unsticking fragments on the surface of the water. **c** Fragments collected one by one on the support grids

Direct deposition of the carbon film: In this case, the carbon film is deposited directly onto support grids covered with a polymer film (collodion, Formvar, or Triafol).

Both films can be used simultaneously or the polymer can be dissolved in order to keep only the carbon film.

The thickness of the carbon film, which can be as thin as 5–10 nm, depends on the amount of carbon evaporated, the distance between the source and target, and the evaporation time. The film quality depends on the nature of the vacuum and the deposition technique (Figs. 2.60, 2.61, and 2.62). The most reproducible method is evaporation under high vacuum, up to rupture of the carbon braid.

The carbon film is chemically inert, supports high temperatures, and provides good flow of electrons and charges during TEM observation.

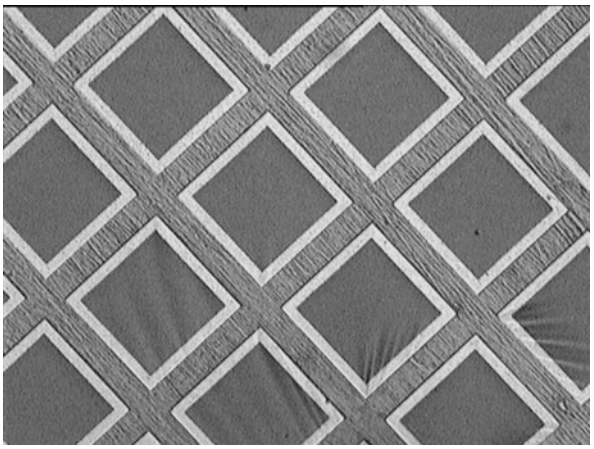

Fig. 2.60 Optical microscope image of a continuous carbon film on a copper support grid, showing no defects. Mesh size: 100 μm (*D. Laub, EPFL Lausanne*)

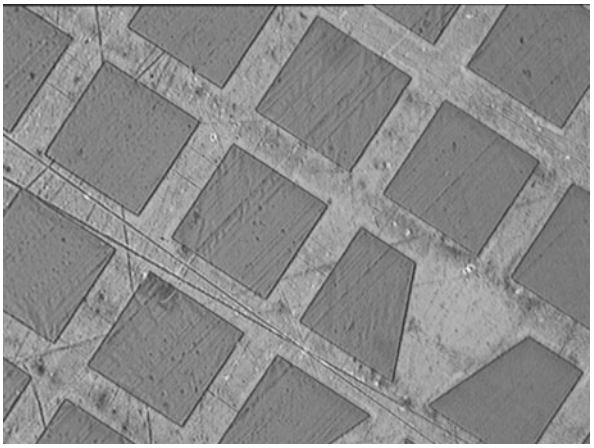

Fig. 2.61 Optical microscope image of a continuous carbon film of poor quality because it presents many streaks and dust. Mesh size: 100 μm (*D. Laub, EPFL Lausanne*)

Table comparing properties of different continuous films

Properties	Collodion	Formvar Triafol	Carbon
Thickness	∼10 nm	∼10–20 nm	∼5–10 nm
Brittleness	Very brittle	Not very brittle	Brittle
Charge effect	Yes	Yes	No
Hydrophobic	No	No	Yes
Easy to make	Yes	No	Yes, sometimes difficult to unstick
Texture	Very fine	Yes	Fine at atomic scale

Fig. 2.62 Low-magnification TEM image of a poor carbon film, resulting in some holes filled in with too much carbon and others where the carbon film is broken (*E. Delain, Microscopie Moléculaire Gustave Roussy, UMR-CNRS, Villejuif*)

13.3 Variant

13.3.1 Ionization of Carbon Films

Freshly prepared carbon films are hydrophobic (or non-polar) and do not allow proper spreading of fine particles in suspension on the water. To remedy this, they must be subjected to an electrical discharge under low pressure (glow discharge) in order to ionize the surface. Similar results can be obtained by exposing them to ultraviolet radiation with a wavelength greater than 360 nm or by letting them age while protected from dust. The advantage of the glow discharge procedure is being able to choose the type of deposited ions (positive or negative) based on the residual gas introduced into the discharge chamber. A rarefied air atmosphere will result in a negatively charged carbon surface. In the presence of pentylamine, the surface will be positively charged (Dubochet method, widely used for spreading out DNA), see the technique for the Dispersion of Fine Particle Materials (Chapter 6, Section 1).

13.4 Advantages

These films are excellent supports for all small fine particles or for crushed, dispersed bulk materials. They are also useful as supports for ultramicrotomy sections or thin slices obtained using FIB.

Collodion film is highly transparent to electrons, but it is very brittle. Formvar film is more resistant, but thicker and therefore less transparent. Only carbon film conducts electrons and is therefore more stable under the electron beam. Furthermore, its texture is very fine, but it can tear under the effect of the electron beam.

13.5 Limitations

Films are rarely perfect and they may also present a texture that makes high-resolution observation difficult. Polymer films are altered or dissolved by alcohols or acetone.

Continuous films are perfectly suited to conventional TEM imaging, but they are not adapted to HREM imaging and EELS analysis, where holey films must be used and observations made through the holes.

For samples requiring a support, grids with ultra-thin carbon films covering the holes should be used.

13.6 Compatible Techniques

FIB, crushing, ultramicrotomy, dispersion of fine particles, negative-staining contrast, positive-staining contrast, and immunolabeling.

13.7 Risks

Theoretical risk of explosion in the case of collodion (nitrocellulose) and chemical risks related to the toxicity of organic solvents. Since carbon deposition occurs under a vacuum, there are risks related to the vacuum.

13.8 Conclusion

Continuous films are essential for the investigation of fine particle materials. They are recommended for any sample that is fragile under the electron beam. Continuous support film grids are also commercially available.

14 Holey Support Films

14.1 Principle

This preliminary preparation consists of making a film with small holes on a grid for supporting small, brittle, or fine particle samples. Holey films must absolutely be used for high-resolution microscopic observations, for EELS analysis, and for

direct observation of frozen hydrated films. The film's holes help to overcome the drawbacks caused by the continuous polymer or carbon support film, such as texture or grain. The sample rests on the edge of the hole and is observed in the hole as if it was floating freely in the microscope.

The polymer film is prepared from collodion, Formvar, or Triafol, pierced with small holes that are reinforced with an amorphous carbon deposit obtained by evaporation under a high vacuum.

Holey films are used as a support for any type of material, in particular for minerals, catalysts, polymers and biological materials, and fine or dispersed particles in liquid solution.

14.2 Operating Mode

14.2.1 Equipment and Supplies

In some cases, it is necessary to use a double-boiler system in order to produce an atmosphere loaded with fine water droplets (at the dew point of water).

14.2.2 Procedure

There are many procedures for making holey films. We commonly use a solution of Triafol (butyril cellulose acetate) in an organic solvent (ethyl acetate or methyl acetate) or a solution of Formvar (polyvinyl formal) in an organic solvent (ethyl acetate or chloroform).

Holes can be made in the film by

- adding glycerol to the initial solution and
- using a glass slide made hydrophobic using an appropriate treatment, in order to create a deposit of very fine water droplets using the dew-point mechanism.

Procedure Using Glycerol

This is a simple, fast method for preparing holey film that consists of adding glycerol to a prepared solution that is composed of a polymer dissolved in an organic solvent. This is all agitated using ultrasound for approximately 1 h, and then a glass slide is dipped into the solution and removed immediately: a film is formed that detaches from the surface of the water. In contact with the water, the holes will form by dissolution of the glycerol fine droplets. Support grids just need to be placed into the holey film and are then removed. After drying, amorphous carbon is evaporated under a secondary vacuum.

There are a large number of possibilities for preparing the initial solutions. The same polymers are used as with continuous films, i.e., collodion or Formvar, and also Triafol in the form of a powder or rigid foil. Triafol has the advantage of being

more resistant mechanically as well as under the electron beam; therefore it is used most often, especially for high-resolution investigations where a very intense and highly focused electron beam is used.

A few examples of making holey films from different initial solutions are provided below.

(A) *Triafol dissolved in ethyl acetate*: Mix 135-ml ethyl acetate and 200-ml Triafol (butyril cellulose acetate) with a magnetic stirrer for several hours. Add a few drops of 87% glycerol (commercially available saturated solution), which is around 0.3 ml. There must not be more than 0.8 ml of glycerol. Stir for 1 h using ultrasound, which will subdivide the immiscible glycerol in the solvent into thousands of tiny droplets that, in contact with water, will give the film its holes.

During this time, clean a glass slide using ultrasound in a degreaser for 10 min, then rinse abundantly in several baths of distilled or demineralized water.

Dry the slide with lint-free paper, always rubbing the slide in the same direction, which will have the effect of aligning the holes (Fig. 2.63).

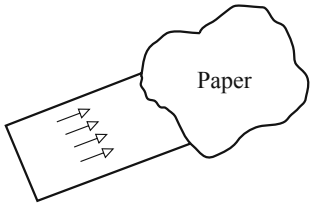

Fig. 2.63 Drying the slide while rubbing it with a paper in the same direction in order to align the holes

Dip the clean, dry slide into the solution containing the Triafol and the glycerol droplets for 10–20 s and pull it out vertically at a rate of 1 cm/s (Fig. 2.64). Allow to dry while protected from dust for 10 min.

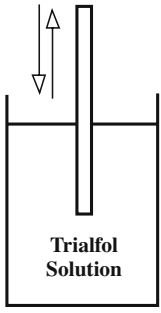

Fig. 2.64 The slice is removed vertically from the Triafol solution at a rate of 1 cm/s (dip-float technique)

Unstick the film obtained on the slide using the dip-float method (see Chapter 2, Section 13). After rubbing the edges of the glass slide using a razor and moistening the film by breathing on it, gradually lower the slide into the water at a low angle (30–45°) (Fig. 2.65).

Let the film float on the surface of the water: Each glycerol droplet dissolves in the water producing a hole in the film.

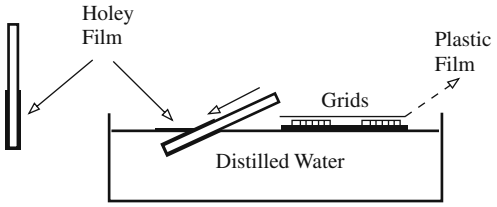

Fig. 2.65 Unsticking and recovery of the holey film (dip-float technique)

Support grids are placed directly on the floating film and recovered by adherence onto a plastic film (parafilm) or paper.

Allow the film to dry for a few hours, protected from dust, and then deposit an amorphous carbon film by evaporation under a high vacuum. The carbon film can be thicker than for continuous films; it reinforces the holey film and makes it conductive, thus preventing charges during the passage of the electron beam.

The number and size of the holes depends on the amount of glycerol in the initial solution. However, when too much glycerol is added, the film may no longer form. When the upper limit is exceeded, once ultrasound agitation is stopped, large drops of glycol fall to the bottom of the flask holding the solution; in this case the film will no longer form.

Also, the holes may not be fully formed. During the carbon evaporation, layers of different thicknesses form in the film, which is very troublesome for observation in the microscope (Fig. 2.66).

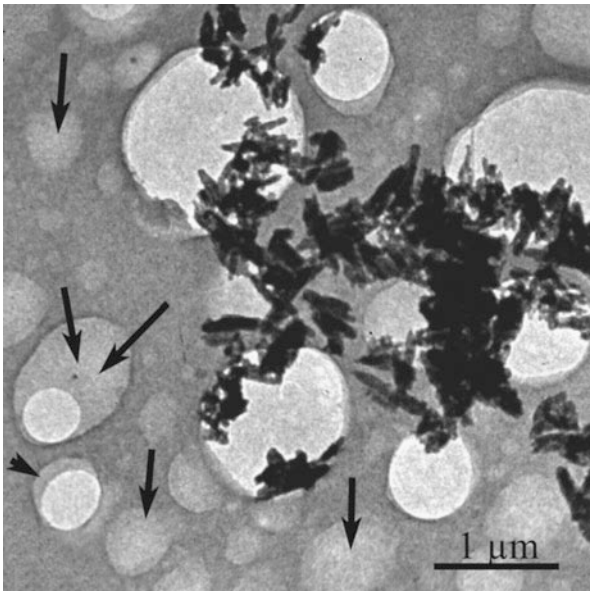

Fig. 2.66 Film with poorly perforated holes indicated by an *arrow*; the perforated holes are *white* (*G. Ehret, IPCMS, Strasbourg*)

The film can be very fine and break during recovery onto support grids; this could be from the solution heating during ultrasound agitation. This agitation must never exceed 1 h.

(B) *Triafol dissolved in methyl acetate*: This variant consists of replacing ultrasound agitation by magnetic stirring over several days.

In this case, another initial solution composed of 0.18-g butyril cellulose acetate in the form of a stiff foil dissolved in 90 ml of methyl acetate (minimum 3 h) is used, to which 0.4 ml of glycerol is added.

This solution is then stirred magnetically for 7–14 days without stopping. Then, the perforated film on the surface of the water is recovered as in the previous example. There are no holes both before and after the time laps between 7 and 14 days; instead, there are bubbles that form, impeding observation in the microscope.

(C) *Formvar dissolved in chloroform*: The initial solution is composed of 0.18 g of Formvar (polyvinyl formal) dissolved in 90 ml of chloroform. A variable amount of 87% glycerol is added to this solution, ranging from 0.01 to 5 ml, which will influence the number and size of holes in the film, from 0.5 to 5 μm.

Procedure Using the Dew Point

This technique consists of making a glass slide hydrophobic, condensing water droplets on it at the dew point, pouring a polymer solution on it, unsticking the film obtained, and recovering it on TEM support grids.

Detailed Technique

A glass slide, after several successive washings, is made hydrophobic by dipping it into a solution of 1% SOFTEX KWO (stearyl dimethyl ammonium chloride). Next, very fine water droplets are made to form on the slide by placing it at the dew point. The size of these droplets depends on the atmospheric humidity and the temperature.

In order to have atmospheric humidity in the workroom, a water bath is run at 333 K for at least half an hour before the start of operations; this also serves to heat the PELEX OTP solution (sodium dialkyl sulfosuccinate), which will help unstick the film at the end of the process.

To be at the dew point, the clean and hydrophobic slide is placed for a few seconds onto a polished copper block cooled for several hours in a freezer at 253 K. In order to obtain holes from 1 to 3 μm, this must occur in a room containing 25–40% humidity and at a temperature between 291 and 296 K, and the glass slide must be left in contact with the copper block for 2–3 s. If this time is exceeded, the size of the holes increases.

The polymer film is made by pouring a polymer solution (0.4% Triafol in ethyl acetate), prepared beforehand, on the slide covered with fine water droplets produced by the dew-point method. The polymer film forms by going around the water droplets. After drying the holey film, the slide can be checked with an optical microscope and it is possible to view the holes and estimate their concentration and sizes. Then the glass slide is dipped into the PELEX OTP solution kept at 333 K in the

water bath, which helps to unstick the film from the water surface. Support grids are dropped onto the floating film and recovered as in the previous case (Fig. 2.65).

This procedure is longer and more difficult than the glycerol procedure, but when all the parameters are controlled, it is possible to obtain holes of a well-defined size. This enables adapting the size of the holes as much as possible to the size of the fine particles to be observed.

After drying the films obtained by one of these procedures, a carbon film is deposited on the support grids by evaporation under high vacuum in order to reinforce the holey film and make it conductive.

14.3 Variant

There are no variants.

14.4 Advantages

These films are excellent supports for high-resolution (HRTEM) microscopy and EELS analysis. The holes help to get around the texture and chemical composition of the film (Figs. 2.67 and 2.68). They are essential for the film of frozen-suspension technique (Chapter 6, Section 2).

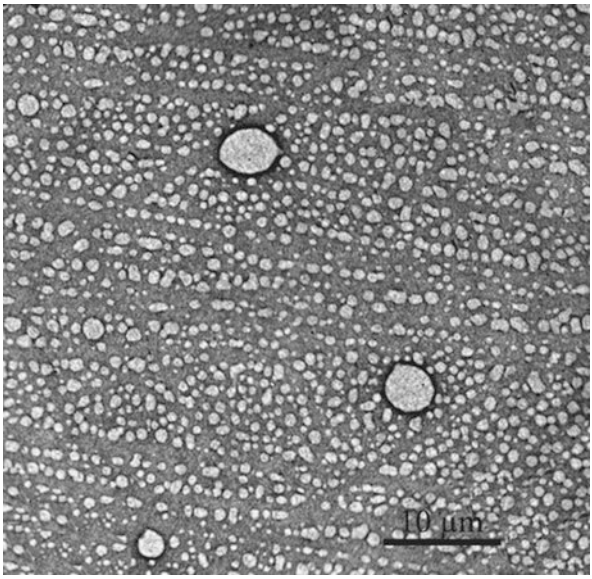

Fig. 2.67 TEM image of a holey film at low magnification. The holes made by the dew-point method are very consistent (*G. Ehret, IPCMS, Strasbourg*)

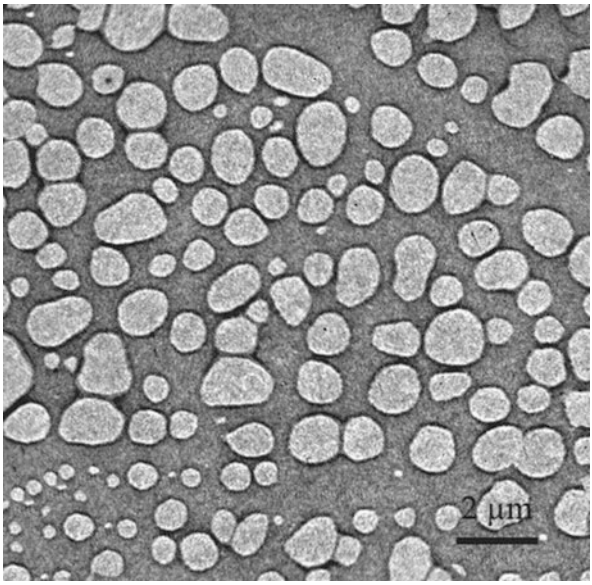

Fig. 2.68 Same holey film at higher magnification (*G. Ehret, IPCMS, Strasbourg*)

14.5 Limitations

The films are rarely perfect; they can present holes that are not thoroughly perforated, which is like performing the observation on a polymer and/or carbon film with an uneven texture (Fig. 2.66).

14.6 Compatible Techniques

FIB, crushing, ultramicrotomy, dispersion of fine particles, and frozen hydrated film.

14.7 Risks

There are chemical risks linked with the toxicity of organic solvents. Since carbon deposition occurs under vacuum, there are risks related to the vacuum.

14.8 Conclusion

Holey films are useful if it is necessary to get around the texture of the film during observation and when the sample must be supported. Holey films are indispensable for investigating fine particles intended for the frozen-hydrated-film technique.

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Chapter 3
Thinning Preparation Techniques

1 Twin-Jet Electrolytic Thinning

1.1 Principle

This technique is used to produce a thin slice (measuring 3 mm in diameter and between 50- and 100-μm thick) without strain hardening, by thinning until a perforation is made in the center. The resulting hole has electron-transparent thin edges.

Twin-jet electrolytic thinning is an anodic electrochemical dissolution technique. The jets provide more action in the center of the sample.

The technique is based on electrolytic polishing of a conductive material; it uses electrochemistry combined with the hydrodynamic effect of an electrolyte jet. A differential dissolution rate occurs on crevices and projections of a rough surface sample, resulting in a flat shiny surface while dissolving the material.

This technique is applied to bulk, compact, usually single-phase metals or semiconductors regardless of their hardness or brittleness. For certain multiphase materials and/or those containing precipitates or segregations, it is sometimes difficult to achieve good thinning conditions.

1.2 Operating Mode

1.2.1 Equipment and Supplies

The equipment consists of an electrical generator or sometimes a potentiostat, which regulates the potential and the dissolution current. The electrolyte is placed in a tank (Fig. 3.1) that must be temperature-regulated because it is generally necessary to work at low temperatures. A cover that holds the polishing cell is placed above the tank. The sample is placed in the specimen holder, leaving both sample faces free, containing an electrical contact (anode) for passing the current (Fig. 3.2). The sample is then placed in the so-called twin-jet electrolytic cell; two nozzles centered on both sides of the specimen provide the electrolyte jets. Each nozzle contains a

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DOI 10.1007/978-1-4419-5975-1_3,

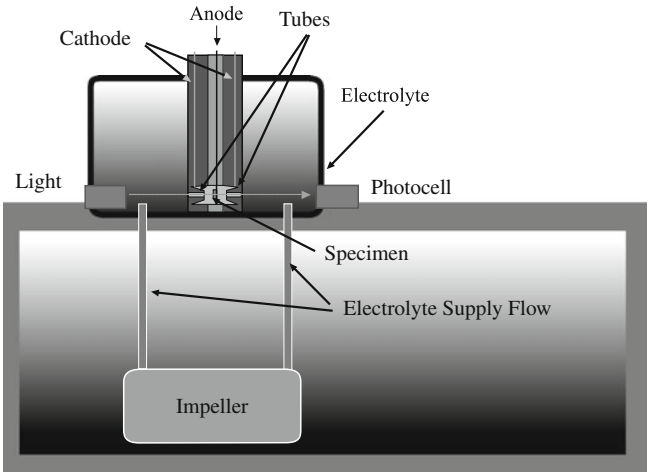
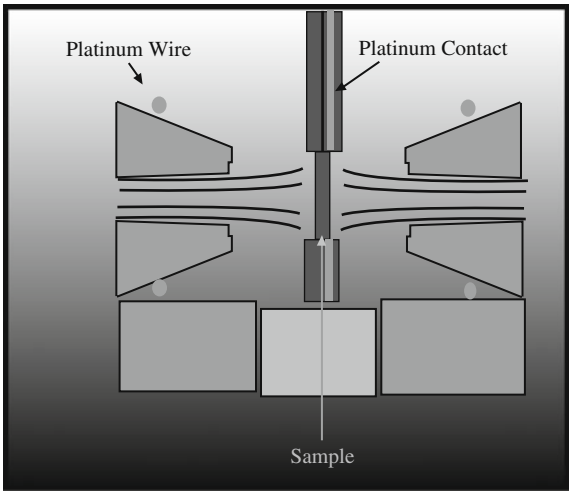


Fig. 3.1 Diagram of the twin-jet electrolytic cell

Fig. 3.2 Details of the electrolytic cell



platinum electrode (cathode) for passing the current. An impeller, with an adjustable flow rate and pressure, is placed in the tank, forcing the electrolyte into the nozzles. The jet force must be perfectly symmetrical so as not to deform the thin edges during etching. A slow jet is used to obtain a broad polish and therefore a large observable surface area. Likewise, the nozzle diameter can be adjusted to obtain more or less broad polishing surfaces depending on the desired effect. A light source and photoelectric cell are placed on both sides of the sample. Once the hole has been made, the photoelectric cell detects the light passing through it and etching is stopped.

Another type of electrolytic cell can be used. This setup is a full bath in a light-proof tank. The nozzles, made from fiberglass braids, are used to conduct a flow of light and recover this signal using a photoelectric cell at the moment of breakthrough in order to stop the process. There are many other types of electrolytic cells, most of which are developed and made in the laboratory. For example, there is a setup where the nozzles are made of two metal tips sheathed in plastic (except the tip), which are placed on both sides of the sample. This results in a preferential dissolution. Since there is no flow, the etching quality leaves something to be desired because the polishing layer is not removed as dissolution occurs.

1.2.2 Procedure

The 3-mm disk is cut using either a precision die (cuts without deformation) or by EDM in a slice with parallel faces, obtained by sawing or mechanical polishing. The electrolytic bath, temperature, voltage, and current conditions to be used are found in specialized books. A current–voltage graph is made on a test sample in order to determine the position of the electrolytic polishing plateau (see Chapter 2, Section 5 on electrolytic polishing). These electrochemical conditions are then applied to the sample to be thinned. The effect of the twin electrolyte jets results in the formation of two dissolution basins that meet in the middle of the sample thickness. Thus, the force of the electrolyte jets thins the polishing layer and causes a hole to form in the center of the slice; however, the jets should not be too strong, otherwise there is etching by direct dissolution instead of polishing. At the end of the thinning process, it is necessary to keep the thick viscous layer and work with low-flow jets in almost stagnant bath conditions in order to ensure thorough finishing and no deformation, as the thin slice is made brittle by the formation of the hole (Figs. 3.3 and 3.4).

When made in this way, the hole (about 100 μm in diameter) is detected by a photoelectric cell receiving a light beam placed on the other side of the sample. The potential, current, and jets are then cut off immediately in order to prevent any dissolution and deformation of the thin edges. This electron-transparent area is observable in the TEM after washing with solvents or water, depending on the case, and drying. Polishing speeds vary widely depending on the material, but usually range between 5 and 50 μm/min.

1.3 Variants

The “single-jet thinning” variant consists of protecting one face of the sample using a non-conductive varnish to prevent the action of one of the electrolyte jets. The thin edge will be at the level of the protected surface, enabling its observation, and no longer in the center of the specimen. This is used when we want to observe a sample processed on the surface by a coating or particular treatment such as implantation or surface segregation.

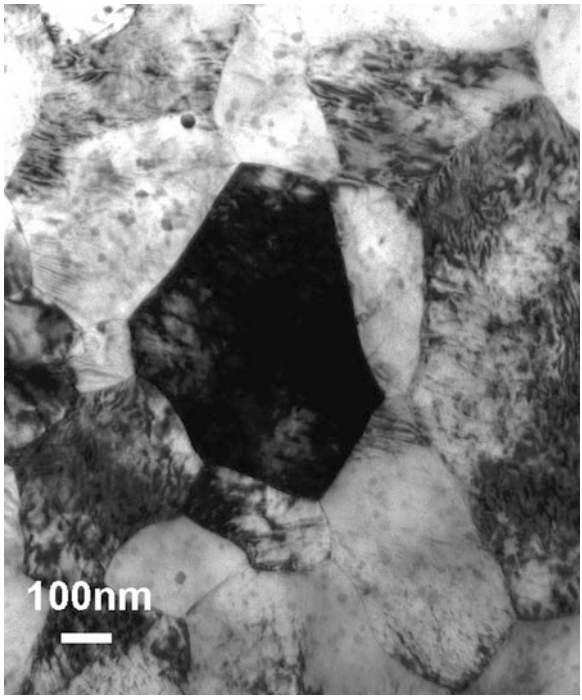


Fig. 3.3 TEM bright field picture of a Steel sample. The black grain at the center is almost completely diffracted (*L. Beaunier, CNRS-LISE-UPR, Paris*)

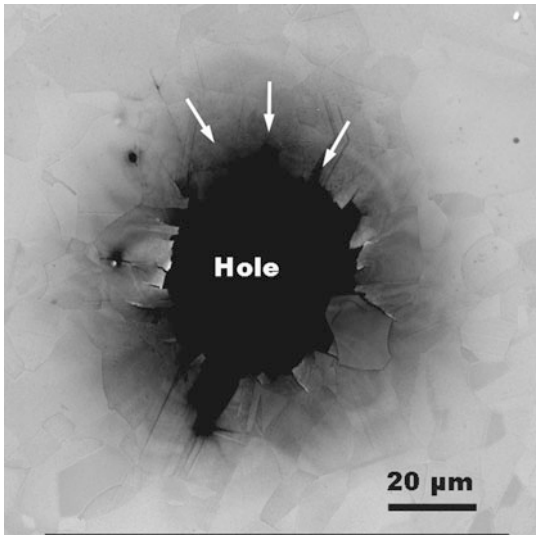


Fig. 3.4 SEM picture of a steel foil. Around the hole, thin electron transparent areas are present (arrows) (*L. Beaunier, CNRS-LISE-UPR, Paris*)

1.4 Advantages

This technique is used to produce thin slices without mechanical damage, regardless of the hardness of the material. The resulting basin is smooth and free of surface roughness. This technique does not generate strain hardening or surface deformation unless the material has internal stresses. Material is not transported from one point to another in the sample. Producing basins makes it possible to keep the outside edges solid and easy to handle.

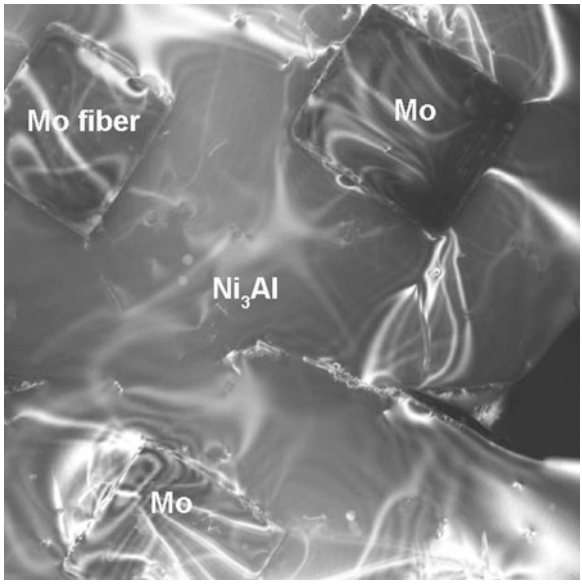


Fig. 3.5 Bright-field TEM image of square Mo fibers in an Ni_3Al matrix, prepared using electrolytic thinning. The Molybdenum fibers and the nickel aluminide Ni3Al have been thinned at about the same thickness. Interface study is then possible. (*D. Laub, EPFL-CIME, Lausanne*)

1.5 Limitations

For inhomogeneous samples containing precipitates or segregations, the difficulty lies in finding the electrolytic solution as well as the potential, current, and temperature conditions. In the case of multiphase materials, there is a risk of selective dissolution (Figs. 3.5 and 3.6).

1.6 Artifacts

There may be dissolution products (oxides, sulfur, etc.) left behind on the sample surface if the bath chosen is incorrect or the polishing conditions are not right. This is rare and only concerns a very small contamination thickness (1–2 nm) on the surface. If this artifact disrupts analysis or observation, a simple light chemical cleaning can be performed, or an ionic thinning, which is more complicated to carry out.

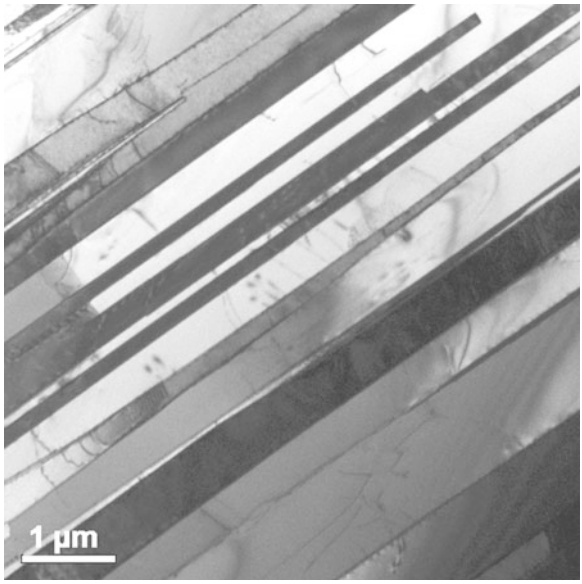


Fig. 3.6 Bright-field TEM image of a TiAl sample, prepared using electrolytic thinning. They are large electron transparent areas, without defects due to the preparation technique (*F. Diologent, EPFL-IMX-LMM. Lausanne*)

1.7 Type of Analysis

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

1.8 Risks

The use of chemicals may create risks of chemical burns and inhalation of noxious aerosols, and in some cases an explosion hazard (perchlorate, etc.).

1.9 Conclusion

This is the ideal technique for all single-phase conducting materials, regardless of their hardness. This is the only technique that generates practically no preparation artifacts and preserves the structures of metals and semiconductors, making it possible to analyze their precipitates or segregants. If it is not possible to find the right electrochemical dissolution techniques, it might be possible to use ion milling instead.

2 Full-Bath Electrolytic Thinning (“Window Technique”)

2.1 Principle

This electrolytic thinning technique is used to produce a thin slice of mechanically prepared metal foils that are approximately 500-μm thick without strain hardening. Dissolution is used to produce a parallel-face thin slice with thin edges directly observable in the electron microscope.

Full-bath electrolytic thinning is an electrochemical dissolution technique.

This is a preparation technique based on electrolytic polishing of a conductive material. It is called the “window technique” because the area of interest is delimited by an isolating film. Outside of this window, the rest of the sample is completely isolated in order to eliminate edge effects. The viscous layer formed on the sample surface leads to a differential surface sputtering, which has the effect of removing bumps and creating a flat shiny surface while the material dissolves.

This technique can be applied to conductive materials such as metals or semiconductors. They must be bulk, compact, and usually single-phased, whether they are brittle or ductile. For certain multiphase materials and/or those containing precipitates or segregations, it is more difficult to find the right thinning conditions.

2.2 Operating Mode

2.2.1 Equipment and Supplies

The equipment comprises an electrical power supply that regulates the potential (voltage) and therefore the dissolution current. A milliammeter and a millivoltmeter could also be used. The equipment is inexpensive and commonly used in laboratories. Machines specially dedicated to electrolytic polishing are commercially available. They can be found in specialized catalogues for polishing equipment or equipment for electron microscopy. Cables are also needed for connecting the electrical power supply to the sample. The electrodes of the polishing cell act as the anode and cathode. The sample is connected to the positive or anodic pole, while the cathodic or negative pole is connected to a plate that constitutes the cathode. The plate must always be larger than the sample (Fig. 3.7) and will be of different compositions depending on the type of metal to be polished (e.g., copper for copper, a stainless steel for steel and ferrous metals, or a platinum grid in other cases). The set is placed in the polishing cell and immersed in the electrolytic solution. Since work is often performed at low temperatures, a cooled beaker placed in a second container filled with ice and salt or a coolant liquid is used. The easiest way to carry out this procedure is to work with a double-walled cell connected to a cryostat where the temperature is regulated. Polishing conditions, including the temperature of the polishing bath, voltage, and cathode material type, are provided either with commercially purchased polishing chemicals (the composition of the etching solution is generally a trade secret) or with solutions cited in reference works. Solutions

are combinations of acids to which thickeners are added to make the solution more viscous (this effect also obtained by lowering the temperature).

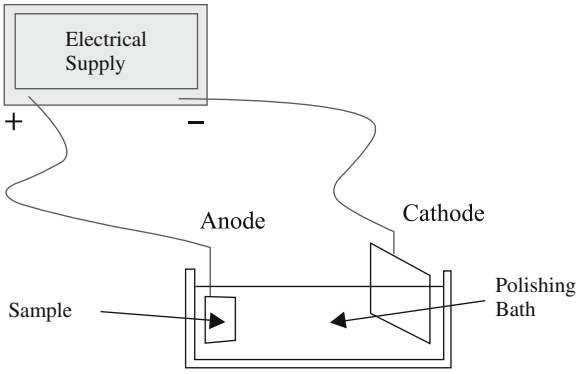


Fig. 3.7 Setup for electrolytic polishing

2.2.2 Procedure

The sample is a foil or thin slice with parallel faces between 50- and 500-μm thick and several tens of millimeters across. In the case of bulk materials, the foil is obtained by sawing and/or mechanical polishing. In metallurgy laboratories, work is often performed on foils of metals that have been laminated to the right thickness and to which the desired thermal treatments have been applied.

Varnish is used to protect the entire surface, except for the area to be thinned, until a hole is made in the sample: this is why it is called the "window technique."

Only the face of the sample to be treated is in contact with the electrolytic bath. The part of the sample covered by the varnish is completely isolated in order to eliminate edge effects. If necessary, the entire rear surface can be protected with a varnish, but this is not mandatory. The sample constituting the anode is then placed in a full bath in the temperature-regulated polishing cell, facing the cathode approximately several centimeters away.

The composition of the electrolytic bath, the temperature, current, and voltage (holding potential) conditions are provided in specialized works, such as the metallurgy or materials handbook. There are many operating conditions for all of the conductor or semiconductor materials.

Since polishing parameters vary with the cell geometry (electrode dimension, relative position with regard to one another, temperature), it is often necessary to make a current–voltage curve in the electrolyte bath. The goal is to precisely determine the position of the electrolytic polishing plateau in the potential range. To do this, the sample is placed in the cell facing the cathode. The sample is always vertical but slightly tilted, face up, so the dissolution layer slides and slowly flows downward. In some rare cases, like copper, the sample must be at the bottom of the cell with the face to be polished facing upward and the cathode placed 1 or 2 cm above it. First, a weak potential is applied and then the dissolution current on the electrical generator is noted (or better still, a milliammeter is used). The value is plotted on graph

paper and then the potential is increased by one unit at a time and so on, until the electrolytic polishing plateau has been passed. The proper polishing voltage value (potential) is close to the upper third of the plateau (Fig. 3.8).

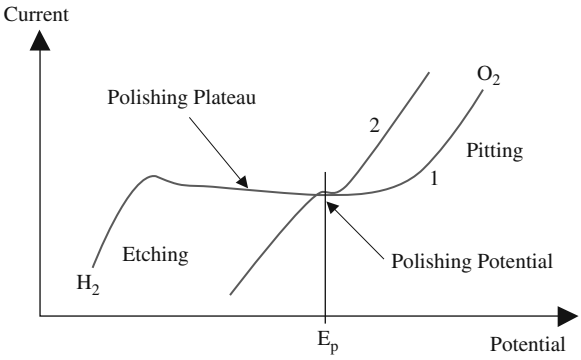


Fig. 3.8 Example of the current–voltage curve

In Fig. 3.8, curve 1 is an example of a perfect polishing curve, whereas curve 2 is the type of curve that is more commonly encountered. At low potentials, there is hydrogen release in the cathodic domain; at high potentials, there is oxygen release in the anodic domain. The plateau represents a potential zone where there is a viscous layer limiting the current and passivating the metal. There is a risk of sputter at the beginning of the plateau and a risk of holes at the end. Once the right polishing conditions are found, work is performed in batches. The sample is placed under voltage in the cell to carry out polishing. The viscous layer formed on the sample surface results in a differential surface sputtering, which has the effect of removing bumps. The end result is a sample with a flat shiny surface, in which the material has been dissolved. The sample is removed from the bath under voltage and washed immediately. The varnish is dissolved, if necessary, and the sample is rinsed with water or solvents before being dried.

For the window technique, dissolution occurs on both sides of the sample, until a hole is made that can be detected with the naked eye.

The steps of the procedure are as follows: The slice is protected around the edges by a varnish; this is the window. The first electrochemical polishing is performed until an edge is etched. Generally, the upper part is etched first, but the edges remain thick. After washing and drying, this part is varnished and then the sample is placed back, upside down. Then a second edge is etched. During this time, the rest of the surface also thins. After washing and drying, the thinned area is varnished, and so on, until the center of the surface has a hole surrounded by a thin edge. The thin edge is ready to be observed in TEM when it looks frayed. A neat edge indicates a thick edge (Fig. 3.9).

The potential is then cut off so that there is no dissolution of the thin edges. The varnish is carefully removed with a solvent and the sample is rinsed and dried. The thin area must then be removed by cutting or with a die (a delicate operation that is not without risk to the sample) and mounted onto a support grid or support ring.

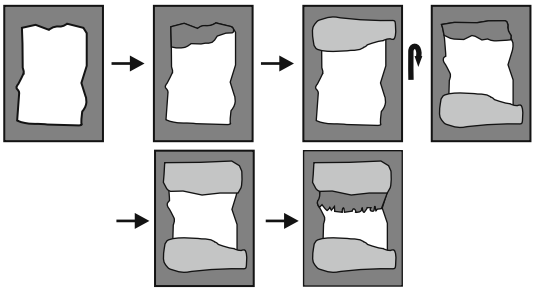


Fig. 3.9 The different steps of the window technique

The sample is now ready for observation in TEM. In other cases, thinning is performed until shards of the sample fall to the bottom of the container; after recovery and mounting on a support grid, they can be observed in the electron microscope (Figs. 3.10 and 3.11).

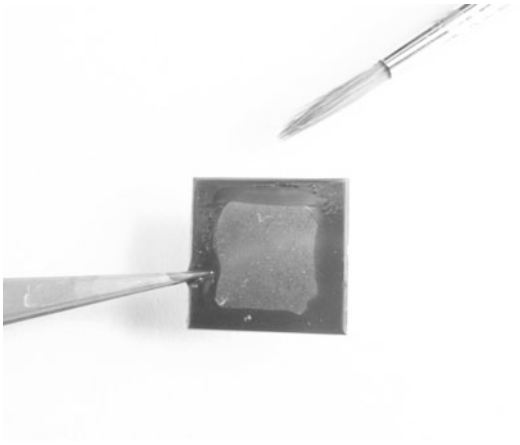


Fig. 3.10 Making the window with varnish (*L. Beaunier, CNRS UPR15 LISE, Université Pierre et Marie Curie, Paris*)

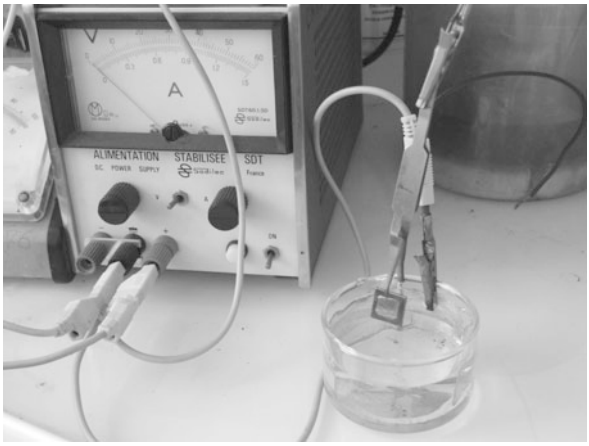


Fig. 3.11 Electrochemical setup for electrochemical polishing (*L. Beaunier, CNRS UPR15 LISE, Université Pierre et Marie Curie, Paris*)

2.3 Variants

2.3.1 Thinning a Single Side

The variant consists of protecting the whole of one side of the sample using a varnish in order to prevent the effect of dissolution of this face. The thin edge of the hole will be at the level of the protected surface, enabling its observation, and is no longer in the center of the specimen.

2.3.2 Thinning a Disk

A sample is taken from the metal to be studied. It is made in the form of a foil by sawing and polishing and is directly cut in the shape of a 3-mm-diameter disk, either using a die or by EDM. Electropolishing is performed on the center of the disk whose edges are protected by a varnish, and as with the window technique, it will produce a hole in the slice in the center of the disk.

2.4 Advantages

This technique can be used for thinning without mechanical damage, regardless of the hardness of the metal or semiconductor. This is the ideal technique for selecting the area to be thinned in order to assess defects visible to the naked eye. The thin slices obtained are thoroughly polished and free of surface roughness. The structure observed is without stress deformation or strain hardening and there is no risk of material transport during thinning.

2.5 Limitations

The difficulty is to find the electrolytic solution, potential condition, current, and adequate temperature for complex or exotic materials. For multiphase materials, there is a risk of selective dissolution. Sampling the thin area is also a delicate step in the technique.

2.6 Artifacts

There may be dissolution products (oxides, sulfur, etc.) left behind on the sample surface if the chemical solution chosen is incorrect or if the polishing conditions are not right. This is rare and at any rate only concerns a very small contamination thickness (1–2 nm). If this artifact disrupts analysis or observation, we can eliminate it by performing a light chemical cleaning (the simplest) or by ionic thinning.

2.7 Type of Analysis

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

2.8 Risks

The use of chemical agents may cause chemical burns and risks of inhalation of noxious aerosols, as well as, in some cases, a hazardous explosion.

2.9 Conclusion

This technique is used for all conductive single-phase materials, regardless of their hardness, and is the preferred technique when the twin-jet technique cannot be applied. It generates practically no preparation artifacts. If this technique cannot be applied, either ion milling, FIB, or the tripod polishing technique can be used.

3 Twin-Jet Chemical Thinning

3.1 Principle

This technique is used to produce a thin slice by thinning the center of a disk measuring 3 mm in diameter and between 50- and 200-μm thick without strain hardening. The resulting basin-shaped perforation has almost parallel faces on the microscopic scale.

The chemical reaction dissolves the sample surface using a polishing effect. The jets have more effect in the center of the sample and end up etching a basin-shaped hole surrounded by thin edges.

As with electrochemical polishing, the most commonly used chemical thinning technique is the "twin-jet" technique. It uses chemical dissolution tied to the hydrodynamic action of the jet, and the technique is used to polish out bumps, eliminate any strain hardening tied to mechanical preparation, and produce a level, polished surface while dissolving the material.

This technique applies to insulating or conductive materials (ceramics, minerals, semiconductors, and metals). These materials must be bulk, compact, and single-phased, regardless of whether they are brittle or ductile. Twin-jet chemical thinning can also be applied to multiphase materials if the chemical bath is chosen correctly; however, for multiphase materials and/or those containing precipitates or segregations, it is more difficult to determine the correct thinning conditions.

3.2 Operating Mode

3.2.1 Equipment and Supplies

The equipment is the same as for twin-jet electrolytic thinning but without the application of current; therefore, the equipment does not include an electrical generator. The electrolyte is placed in a cell, which must be temperature-regulated because work must generally be done at low temperatures. Above the tank is a cover that holds the polishing cell (Fig. 3.12). The sample is placed in the specimen holder,

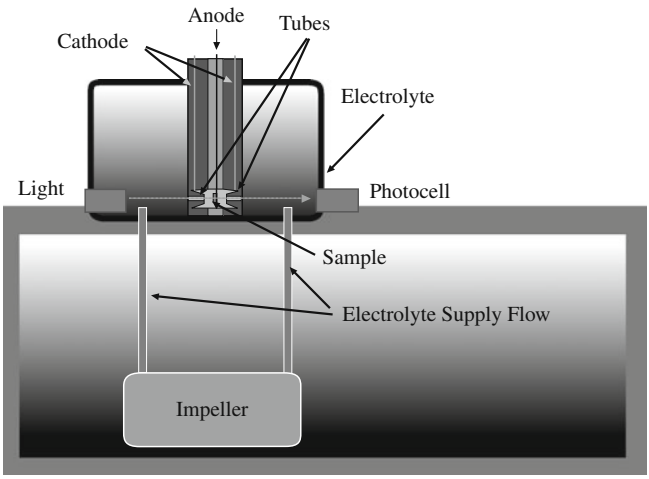


Fig. 3.12 Diagram of the twin-jet chemical cell

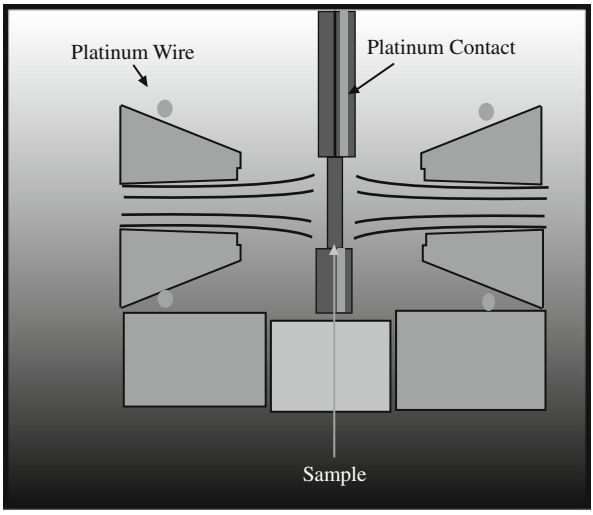


Fig. 3.13 Details of the electrolytic cell

leaving the two faces of the sample free. The holder is then placed in the "twin-jet" cell. Two jets centered on each face of the object, aimed toward each other, provide the flow of the electrolyte jets (Fig. 3.13). An impeller placed in the tank forces the electrolyte into the tubes at an adjustable flow rate and pressure. The diameter of the tubes is adjustable in order to control the breadth of the surface areas to be polished, depending on the desired effect. A light source and photoelectric cell are placed on both sides of the sample. Once a hole is formed in the center of the specimen, the photoelectric cell detects the light passing through it and polishing is stopped.

3.2.2 Procedure

The sample is a disk 3 mm in diameter and between 50- and 200-μm thick. For conductors, the sample is ground to a thin slice with parallel faces obtained by wheel sawing or mechanical polishing. The chemical solution and temperature conditions are chosen from specialized works on electron microscopy. The slice is then placed onto the specimen holder for thinning. The jets, which are facing each other, hit each side of the slice and form two basin shapes in the center of the specimen, thereby creating a hole that is detected by the photocell. The sample is rinsed with water or solvents and dried. It is then ready for observation in the microscope.

3.3 Variants

The single-jet thinning variant consists of protecting one face of the sample using a non-conductive varnish in order to prevent the action of one of the jets. The thin edge around the hole will be at the level of the protected surface and no longer in the center of the specimen. This is used to observe a sample processed on the surface by a coating or particular treatment such as implantation or surface segregation.

3.4 Advantages

This technique is used to obtain a basin-shaped thinning, down to a hole in the center of the specimen, without mechanical damage, irrespective of the material hardness. The resulting basin is polished and free of surface roughness. The observed structure is without strain hardening or stress deformations. There is no material transport in the sample. Producing basins makes it possible to keep the outside edges solid and to easily handle the specimen with tweezers or suction.

3.5 Limitations

The difficulty lies in finding the adequate chemical solution and correct temperature conditions for complex or exotic materials. In the case of multiphase materials, there is a risk of selective dissolution. It is also possible to reveal crystallographic planes with low indices (dense crystallographic planes).

3.6 Artifacts

In all cases, there may be dissolution products remaining (oxide, sulfur, etc.) on the surface if the chemical is not appropriate or if the right polishing conditions are not met, but this is rare and only concerns a very thin contamination layer. If this artifact disrupts analysis or observation, a light cleaning using ionic thinning is performed.

3.7 Type of Analysis

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

3.8 Risks

They may be a risk of chemical burns, inhalation of noxious aerosols, and rarely an explosion hazard.

3.9 Conclusion

This is the ideal technique for all single-phase insulating materials, regardless of their hardness. It generates practically no preparation artifacts. It is the ideal technique for investigating dislocations. If this technique cannot be applied, then either the ion milling, FIB, or tripod polishing technique should be used.

4 Full-Bath Chemical Thinning *("Window Technique")*

4.1 Principle

The goal of this preliminary preparation technique is to obtain surface etching with a polishing effect. It is used to produce a thin slice of approximately 100-μm-thick foils prepared mechanically, without strain hardening. Dissolution is used to produce thin edges with parallel faces directly observable at the TEM scale.

Full-bath chemical thinning is a chemical dissolution technique based on the same principle as chemical polishing, in that the layer formed on the sample surface results in sputtering of the relief, which has the effect of removing bumps and producing a shiny planar surface.

Full-bath chemical thinning (or the "window technique") was more widely used in the past, before the development of twin-jet equipment. It is based on the chemical polishing of a conductive or insulating material. The area of interest is delimited by an isolating film, giving the so-called window technique its name. The uncovered part of the sample is completely isolated in order to eliminate edge effects, and the specimen is left in the full bath to undergo chemical etching until a hole forms. The technique is used to polish out bumps, eliminate any strain hardening tied to mechanical preparation, and produce a polished planar surface while dissolving the material.

The technique applies to insulating or conductive materials (ceramics, semiconductors, minerals, and metals). These materials must be bulk, compact, and single-phased, regardless of whether they are brittle or ductile. The technique is also applied to multiphase materials, if all of the phases can be dissolved at the same rate. However, for multiphase materials and/or those containing precipitates or segregations, it is more difficult to find the correct thinning conditions.

4.2 Operating Mode

4.2.1 Equipment and Supplies

The equipment consists of a chemical etching bath in an adjustable-temperature glass or Teflon vessel. The sample is placed in a full bath in a beaker, which may or may not be temperature-controlled. Only the area of the sample to be treated is in contact with the chemical bath; a varnish protects the edges. While the process is underway, it should be monitored using a stereoscopic microscope. The sample is laid at the bottom of the chemical bath using a pair of tweezers. The type of etching solution used depends on the material's chemical composition; they are generally acids or bases. This information is provided in specialized books or through knowledge of chemistry.

4.2.2 Procedure

The sample is a foil or thin slice with parallel faces between 50- and 500-μm thick and several tens of millimeters across, made either by mechanical polishing or sawing. Varnish is used to protect the perimeter of the sample surface, except for the area to be thinned (Fig. 3.14).

The sample is placed directly in the vessel containing the bath for chemical etching (Fig. 3.15). The chemical layer formed on the sample surface leads to a sputter of the relief, which has the effect of removing bumps, resulting in a flat surface while reducing the thickness of the material.

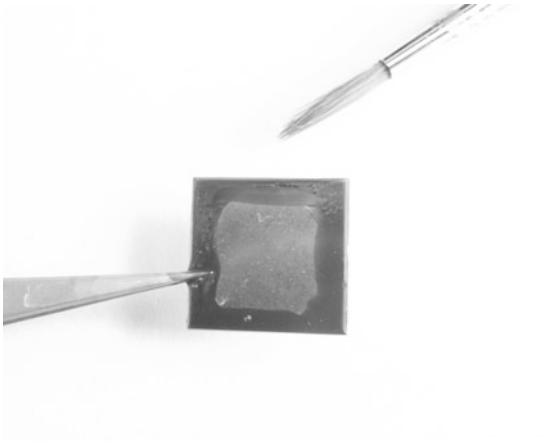


Fig. 3.14 The edges of the sample are covered with varnish (*L. Beaunier, CNRS UPR 15 LISE, Université Pierre et Marie Curie, Paris*)

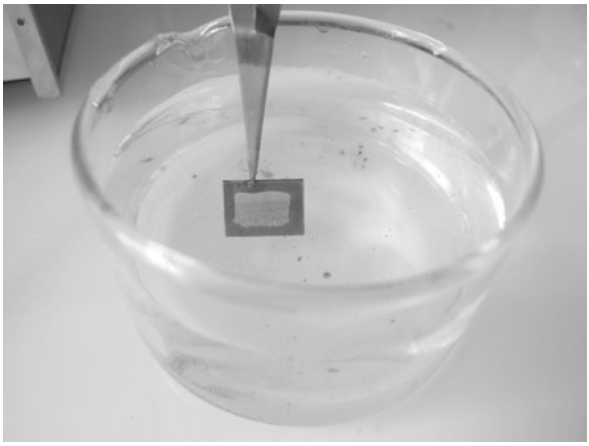


Fig. 3.15 Etching in the chemical bath (*L. Beaunier, CNRS UPR 15 LISE, Université Pierre et Marie Curie, Paris*)

For this technique, dissolution occurs on both sides of the sample until a hole is made that can be detected with the naked eye.

The steps of the process are as follows: In the first step of the chemical thinning, the slice is protected around the edges by a varnish; this is the window. The first polishing is performed until one side is etched. Generally the upper part etches first, but the edges remain thick. After washing the sample with water or solvents and then drying it, this first part is varnished and then the sample is put back in the solution upside down. Then, a second edge is etched. During this time, the uncovered surface thins. After washing and drying, the thinned area is varnished again and so on, until the center of the slice has a hole with a thin edge. The thin edge is ready when it looks frayed (Fig. 3.16). A neat edge means it is still too thick.

If necessary, the varnish is carefully removed with a solvent. The sample is rinsed and dried. Then the thin area must be sampled by cutting with a razor blade, scissors,

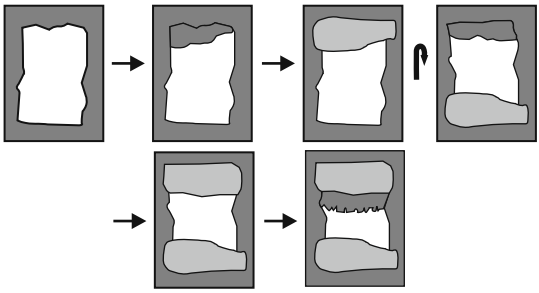


Fig. 3.16 Window technique

or a punch (a delicate operation that risks breaking the sample). The thinned area is stuck onto either a support grid or support ring, so that it can be observed in the microscope. In other cases, thinning is performed until shards of the sample fall to the bottom of the container; after retrieval and mounting on a support grid, they can be observed in the microscope.

4.3 Variants

The variant consists of protecting all of one whole face of the sample using a varnish to prevent this face from dissolution. This variant is used to investigate surfaces with a coating or a treatment (implantation, segregation, etc.). Therefore, the matrix is dissolved by contact with the chemical bath, until the treated face is eventually exposed. The thin edge will be at the level of the treated surface, enabling its observation in the electron microscope. In this case, the thin edge to be observed will no longer be in the center of the specimen.

4.4 Advantages

This technique can be used for thinning without mechanical damage, regardless of material hardness. This is the ideal technique for selecting the area to be thinned in order to observe defects visible to the naked eye. The resulting slice is thoroughly polished and free of surface roughness. The structure is without strain hardening or stress deformations. There is no risk of material transport during thinning.

4.5 Limitations

The challenge lies in finding the right chemical solution and temperature for complex or exotic materials. For classic materials (metals, semiconductors, ceramics, minerals, etc.), data are available in the literature. Sampling the thinned area remains a delicate step. There can be selective etching, e.g., on precipitates, or it can

reveal crystallographic planes of low indices corresponding to dense planes. Unlike the twin-jet technique, which produces a precisely located hole, thinning occurs anywhere on the slice.

4.6 Artifacts

In all cases, there may be dissolution products remaining (oxide, sulfur, etc.) on the surface if the bath is not appropriate or if the right polishing conditions are not met, but this is rare and only concerns a very thin contamination layer. If this artifact interferes with analysis or observation, it can be eliminated by performing a soft cleaning using ion milling.

4.7 Type of Analysis

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

4.8 Risks

There are risks of chemical burns, the inhalation of noxious aerosols, and rarely an explosion hazard.

4.9 Conclusion

This technique is used for all insulating single-phase materials, regardless of their hardness. It is the preferred technique when the twin-jet technique cannot be applied. It generates practically no artifacts. If this technique cannot be applied, ion milling, FIB, or the tripod polishing technique can be used.

5 Ion Milling

5.1 Principle

This technique is used to thin samples down to electron transparency. It is generally used on a 3-mm-diameter disk, on the order of between a few microns to tens of microns thick. It is also used for final thinning of samples prepared using the tripod polishing technique.

The thinning technique consists of abrading the sample using ionic bombardment (ionic abrasion and ionic cleaning).

The production of a beam of ions that is accelerated and directed toward the center of the sample is used to lift atoms from the surface. It is used to reduce bumps, and it removes any strain hardening tied to a preliminary mechanical preparation. Machining with the ion beam can be on a plane-longitudinal cut, on a cross section, or on a wedge slice (tripod).

Any type of compact, single-phase, or multiphase material can be thinned. Porous or fine particle materials must be infiltrated or embedded beforehand.

5.2 Operating Mode

5.2.1 Equipment and Supplies

This technique requires special dedicated equipment, consisting of a chamber under a vacuum of approximately 10^{-3} Pa, containing one or two guns (Steigerwald or Penning) that generate argon ions (Ar^+). The guns are aimed at either a single side or both sides of the sample. The chamber has a porthole for observing the sample with an optical microscope or a CCD camera during ion milling, and allows as well to visualise the ion beam(s) when centering them (in translation and focusing).

The beams of charged ions are accelerated by a voltage on the order of between a few electron volts and a few kilovolts. The beams are adjustable in pitch from 90° to 0° with regard to the horizontal plane of the sample.

Part of the beam is not ionized and therefore contains neutral atoms. The rotating specimen holder can be cooled with water or liquid nitrogen. An optical microscope

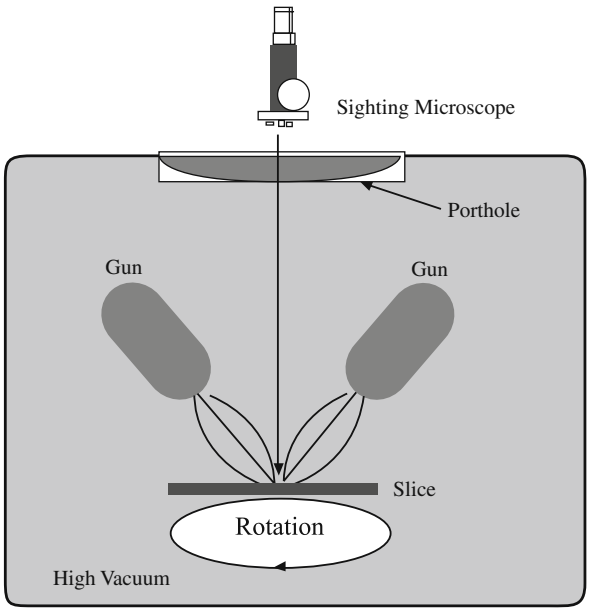


Fig. 3.17 Ion-thinning principle

may be equipped with a camera for monitoring the machining of the sample. When a hole is made, milling can be stopped when it is detected, either by a light beam received by a photocell or by a Faraday cage under the sample that receives the ion beam (Figs. 3.17 and 3.18).

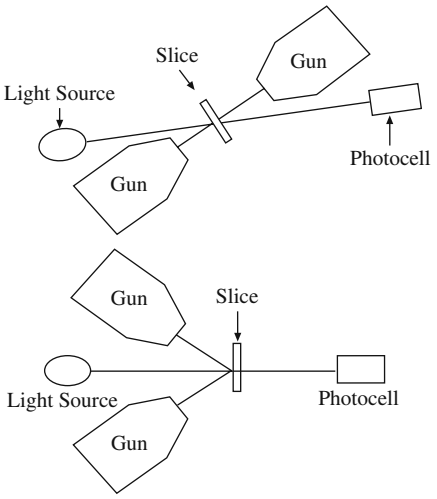


Fig. 3.18 Gun arrangements

Thinning is automatic and easy to implement. *Caution*: Ionic cleaning can heat samples to temperatures of several hundreds of degrees Kelvin for beams at angles greater than 10° with regard to the surface of an uncooled specimen holder.

Some devices are used to perform ionic polishing of samples with large surface areas and even to make cross-sectional cuts for scanning electron microscopes (Fig. 3.19).

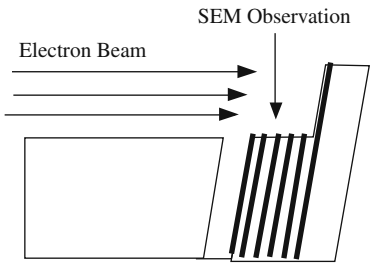


Fig. 3.19 Cross-sectional cut for the scanning electron microscope

5.2.2 Procedure

Generally, the 3-mm disk is prethinned into a dimple (either by dimpling or by electrochemical polishing or twin-jet chemical polishing). However, if a cross-sectional cut is needed for observing multilayer samples in cross section or simply for observing defect or composition gradients, the sample is first prepared using either the sandwich technique or a variant of it.

When stacking more than two samples, there are several preparation steps: stacking the flat samples under a press to make a stack more than 3-mm-thick, cross-sectional grinding (by the trench) of the 3-mm-diameter multiple stack, cutting the 3-mm bar with a wheel saw or wire saw, polishing, dimpling, and ion milling.

The glues and resins used for preparing samples must be chosen specifically in order to prevent differential thinning rates even though the low-angle technique reduces these effects.

Ionic Thinning

The sample is placed on the specimen holder by either hot or cold gluing, or it is clamped. The sample is centered very carefully using a stereoscopic microscope. The acceleration voltage of the ion beams is regulated, as is their sharpness and angle. Charts are used to regulate the acceleration voltage of the beams and the induced current. Working at low energy will induce fewer defects but effectiveness is lower and therefore thinning time is longer. The beams (on the order of 1 mm in diameter) are then focused carefully onto the center of the sample (Fig. 3.20). The specimen is rotated in order to ensure continuous cylindrical machining. It is also possible to work with a sectorial rotation (adjustable between 0° and 120°) to shield certain parts of the sample from ionic abrasion. Particularly, the beam must be prevented from being in the axis parallel to the gluing area when working on cross-sectional cuts of samples glued face-to-face (Fig. 3.21). Either one or two ion beams on one side of the sample are used to polish just one face, or one beam on each side of the sample polishes both faces simultaneously. In this case, both polishing dimples meet at the center of the sample thickness. Piercing of the sample can be detected either with a microscope or with a photocell receiving a light beam. The sample is then ready for observation in the microscope.

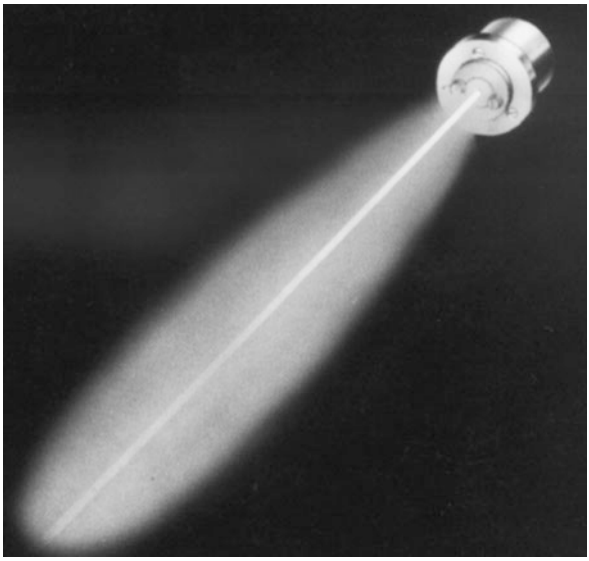


Fig. 3.20 Shape of an ion beam

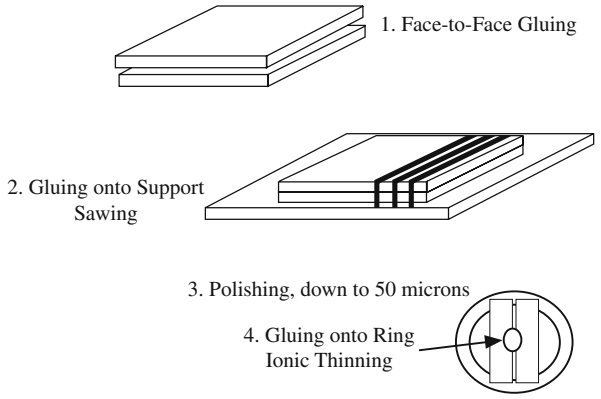


Fig. 3.21 Preparing cross-sectional sample

The incidence angle of the guns is adjustable from 0° to 90° depending on the ion mill. A high incidence angle, near perpendicular to the thin slice, enables rapid thinning but induces significant damage (ion implantation, thermal effects, chemical diffusion, etc.) and residual surface roughness due to sample inhomogeneity. Newer ionic thinning devices can etch samples at very low incidence angles (<10°), producing genuine surface polishing (Fig. 3.22). This yields a thin, broad area (50–500 μm) of great quality. Additionally, latest-generation guns allow for large flow rates and therefore significant abrasion speeds, on the order of a half hour, instead of the tens of hours required in the past.

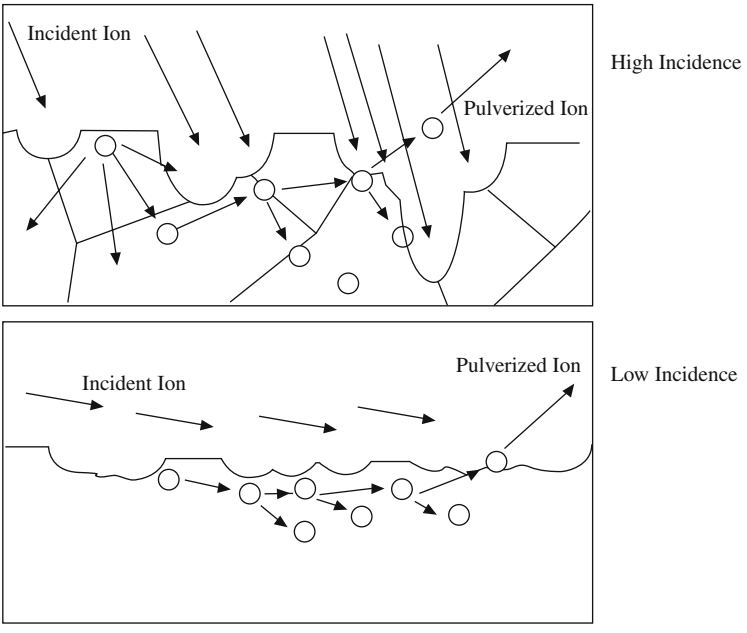


Fig. 3.22 Influence of the ion beam incidence angle with regard to the sample surface roughness

Milling can take between a few minutes and 24 h, depending on the effectiveness of the ionic sources, the material type, the sample thickness, and the equipment technology, which depends on the operating parameters (voltage, current, and gun incidence angles).

The surface can be machined through scouring. First, large beams at high incidence angles are used to obtain a dimple that is already significantly thinned. Then, very sharp beams and low incidence angles are used to mill the central area of the sample and complete the milling process.

One ideal condition is to use ionic thinning on very thin flat slices (<20 μm). These are thinned with dimpling down to ≈5 μm in the center, on just one face, in order to obtain as wide a dimple as possible. Therefore one flat face remains that will be machined by the beams. Beams at incidence angles less than 10° are used, and then the angle is reduced further, down to 1–2°. Under these conditions, it must be noted that the geometric settings are of utmost importance because work is being performed at the micrometric scale. The beams are almost parallel to the surface and the edges of the sample could mask the beam (Fig. 3.23).

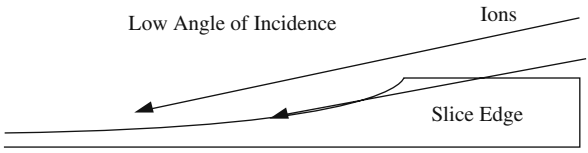


Fig. 3.23 Geometry under low incidence angle

Monitoring with a stereoscopic microscope or a camera can help to see the remaining roughness on the sample surface, so that the milling parameters can be modified if necessary. This technique is used for cross-sectional cuts containing stacks of different materials such as glue–polymer–metal–ceramic in a single sample. Figures 3.24 and 3.25 show how ion beams angled at 10° can induce roughness when thinning a steel specimen. This roughness is due to inhomogeneities (hard and soft phases) in the metal.

Figure 3.26 shows a complex sample milled first at an angle of 10°, enabling fast thinning, but creating surface roughness. Finishing will then be at a low angle, in order to flatten the surface.

Some ion milling devices allow for the diversion of charged ions by applying a retarding field. This extension was developed to eliminate preferential sputtering and ion shadowing at low-angle bombardment. The incident ion beam is bent over the sample surface with a retarding field ranging from 0 to 2.5 keV. This method also eliminates preferential etching due to grazing incidence and results in the neutral atoms continuing on their straight-line path. The stage and the retarding field are polarized in order to precisely center the scouring point of the ions or neutral atoms. This method is used to regulate the angle of incidence of the beams on the surface without being impeded by the thick edges of the sample, as with a direct beam in a low-angle position.

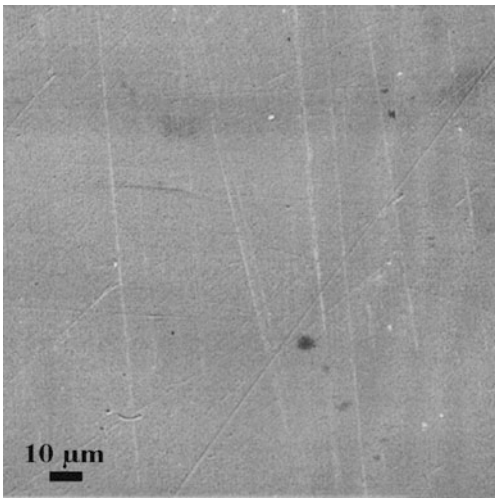


Fig. 3.24 Surface state of a Z39 steel after mechanical polishing. Scratches remain on the surface

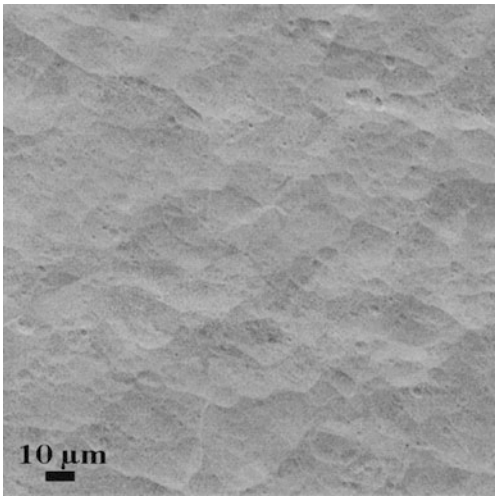


Fig. 3.25 Same sample as Fig. 3.24 after partial ion bombardment, showing surface roughness mainly induced by the ion milling process

– In normal operation, the ions and neutral particles will hit the sample.
– By deflection and the retarding field, we can divert the ions and only the neutral particles will interact.
– By translation, we can then focus just the ions (Fig. 3.27).

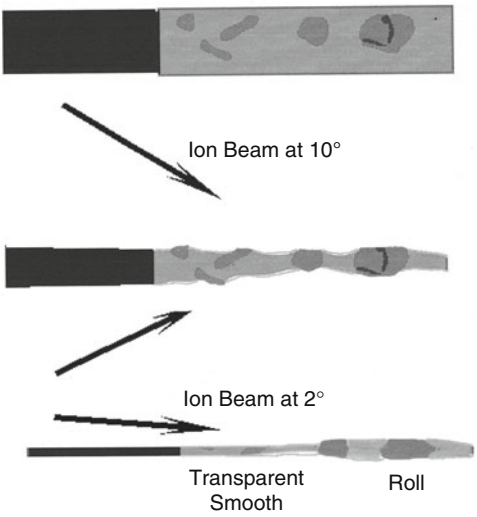


Fig. 3.26 Example of a possible milling process for a multiphase material

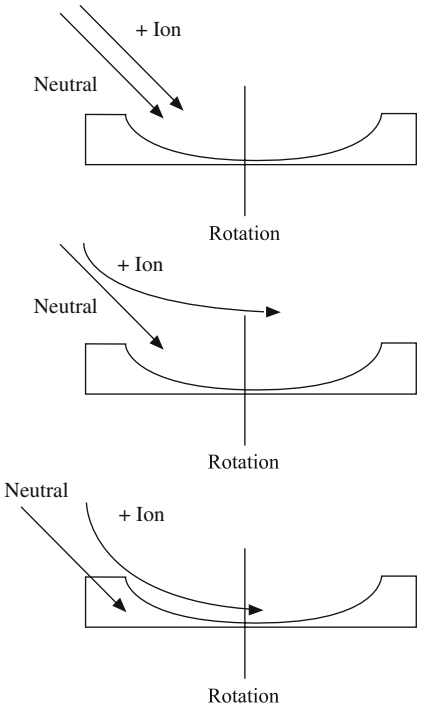


Fig. 3.27 Principle of deflecting the ion beam

5.3 Variants

In some cases it is necessary to work in a reactive atmosphere using chemical-assisted ion beam etching (CAIBE). Some samples cannot be etched easily with argon ions. Using a tube, a gaseous chemical atmosphere is delivered to the sample surface. It reacts with the surface of the material and generates a reaction product that will be etched by the ions. Working in a reactive atmosphere accelerates or improves ionic abrasion. For example, iodine is used for InP.

Material example: Co/Ir multilayer (Figs. 3.28 and 3.29) deposited by Epitaxia using molecular jets onto a buffer layer of Ir on MgO substrate.

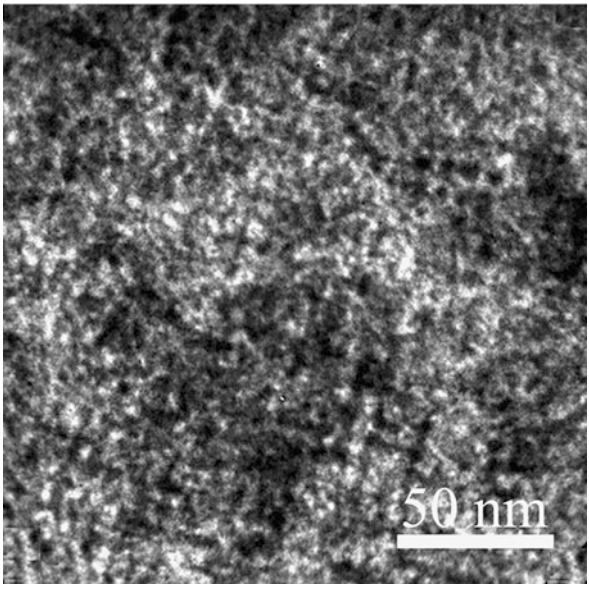


Fig. 3.28 Plan view obtained using the mechanical polishing preparation technique, then thinned using ion milling. We can make out marbling at 5.7 nm, which corresponds to the interference between planes (200) of the Ir buffer layer with those of the superlattice constituting the multilayer (*C. Ulhaq-Bouillet, IPCMS, Strasbourg*)

5.4 Advantages

This technique can be used for thinning without strain hardening, regardless of the nature, hardness, and brittleness of the material. It is very well adapted to preparing samples in the form of a sandwich (cross section). Mixed–composite materials can be thinned easily.

5.5 Limitations

There is a risk of material transport (re-deposition) under the ion beams from one point to another on the prepared surface (atoms that have been lifted up fall back

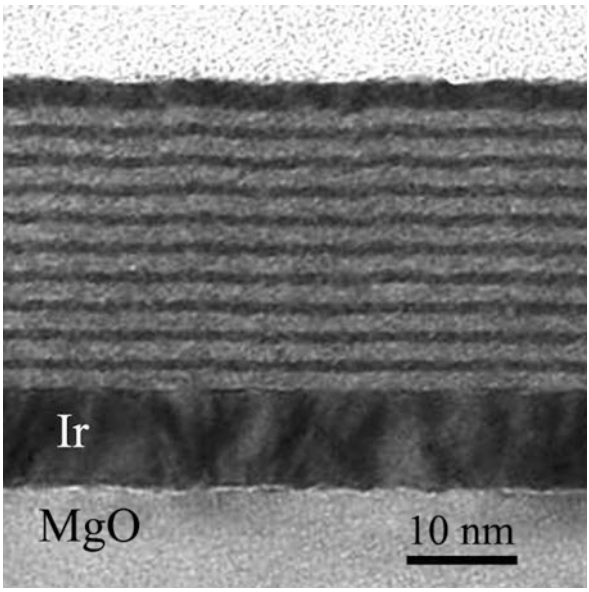


Fig. 3.29 Cross section obtained using the sandwich preparation technique, then thinned using ion milling. The cross-sectional cut is used to analyze the stacking of different layers as well as their interfaces. The MgO substrate and the 10-nm-thick Ir buffer layer can be clearly seen, as well as the alternation of the Co and Ir layers (the contrast between layers is determined by the atomic number of the elements: Ir>Co and therefore is darker) (*C. Ulhaq-Bouillet, IPCMS, Strasbourg*)

down on the sample), sputter (the effectiveness of etching depends on the chemical nature of the material), implantation of argon ions, chemical diffusion, thermal damage, and irradiation damage. In order to minimize these drawbacks, it is possible to cool the sample and reduce both the angle of incidence of the guns and the acceleration voltage.

5.6 Artifacts

The major problem is the implantation of argon ions in the sample with a possible structural change: amorphization and phase transformation. The importance of the implantation depends heavily on the acceleration voltage. Low voltages produce practically no ion implantation and reduce surface amorphization.

5.7 Type of Analysis

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

5.8 Risks

Risks are related to the use of reactive chemicals and liquid nitrogen for cooling.

5.9 Conclusion

Among all the techniques, ion milling, along with focused ion beam thinning (FIB), can be used to prepare the widest range of materials, with the exception of very soft materials. This technique is often the final step in addition to other techniques.

6 Focused Ion Beam Thinning (FIB)

6.1 Principle

The purpose of this technique is to machine a thin lamella (thin slice) of a constant thickness, on the nanometric scale, in a precise area of a sample.

It first consists of depositing either a metal layer or a carbon layer to protect the material surface during ion milling, and then using an ion beam (Fig. 3.30) to machine two parallel trenches on both sides of the sample area to be observed. The resulting wall constitutes the parallel-sided thin slice (Fig. 3.31). The FIB can be equipped with an internal micromanipulator for extracting the lamella after machining.

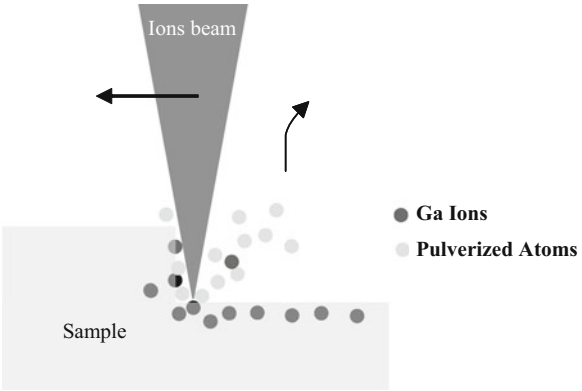


Fig. 3.30 Diagram of ionic abrasion of a sample, side view

This technique is used to prepare TEM lamella of any type of single-phase or multiphase material, of any hardness, with the exception of very soft materials or those with a liquid solution. It is also a great tool for SEM sample preparation in materials science and biology, especially for 3D reconstruction. There are three ways to obtain a thin slice:

1. *Internal extraction method:* This consists of using a micromanipulator to weld the thin slice, inside the FIB, to a special support adapted to the TEM. The lamella is held in such a way that it can then be moved to undergo final thinning, and then taken out of the FIB to be observed in the TEM (Fig. 3.32).

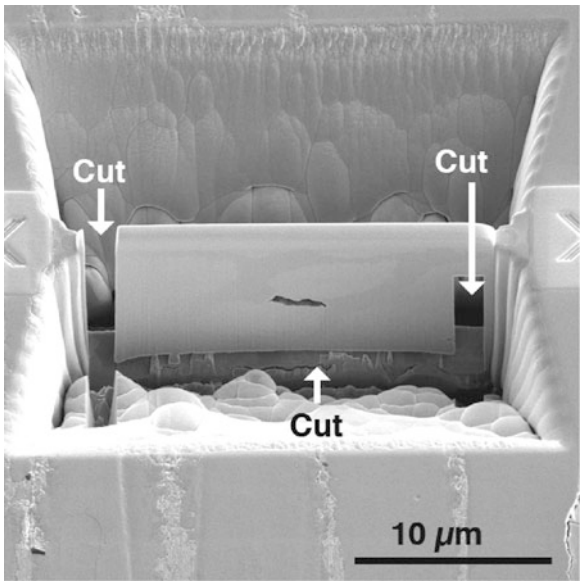


Fig. 3.31 Thinning a thin lamella of constant thickness, which will then be extracted from the bulk material. SEM image in secondary electrons (*F. Bobard, EPFL-CIME, Lausanne*)

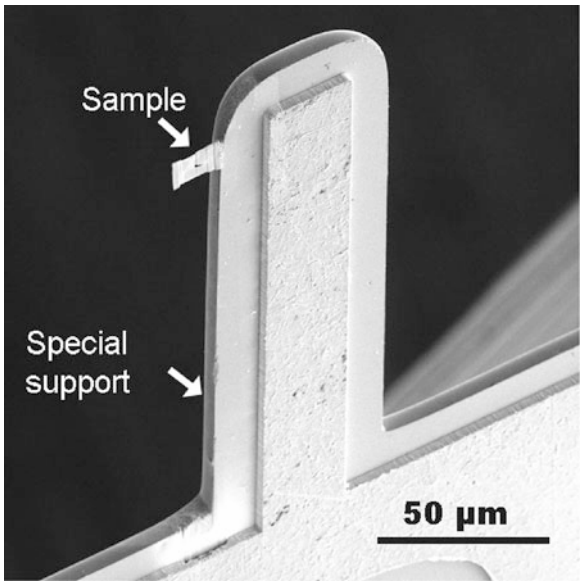


Fig. 3.32 SEM secondary-electron image of the lamella welded to the special support (*F. Bobard, EPFL-CIME, Lausanne*)

2. *External extraction method:* This consists of placing the lamella onto a TEM grid coated with a carbon film (Fig. 3.33). In order to make this sampling, the sides of the slice, as well as the base, are cut using the ion beam to free the thin slice, then the sample is removed from the FIB and the lamella (still in its original placement) is transferred to the TEM grid, using a fine electrostatically charged

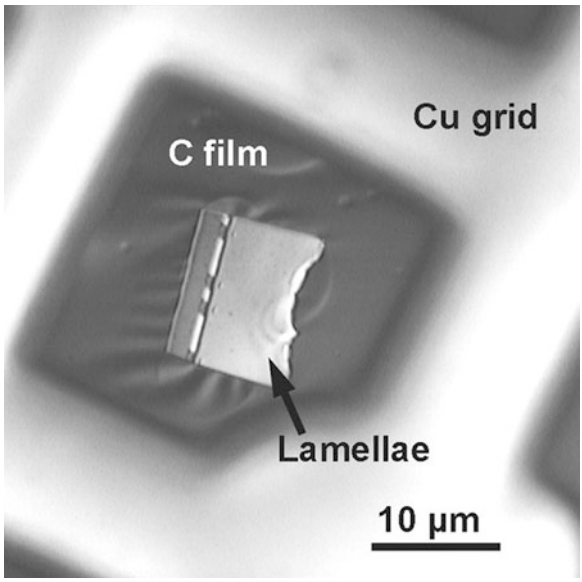


Fig. 3.33 Optical microscopic image of a lamella sampled using the external extraction method, placed onto a support grid coated with a layer of carbon (*F. Bobard, EPFL-CIME, Lausanne*)

glass needle. The thin slice extracted from the bulk material is then ready for TEM observation.

3. *H-bar method:* This consists of thinning a thin slice on a material that has been pre-thinned by mechanical polishing to a thickness of approximately 30 µm, then mounted on a special support (Fig. 3.34). The slice is not extracted; it remains connected to the bulk material.

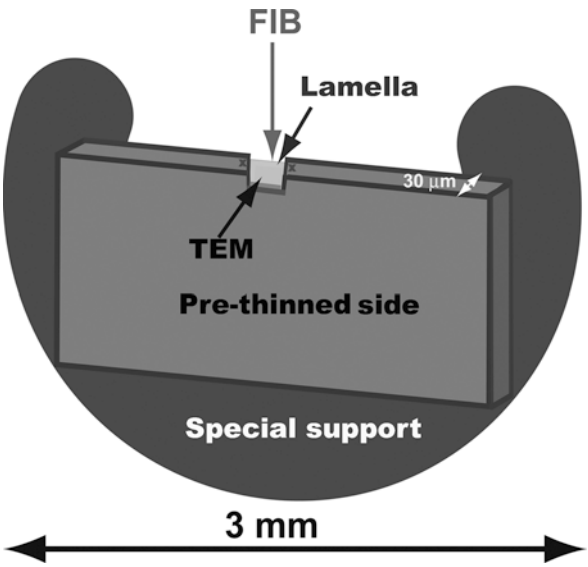


Fig. 3.34 Diagram of a material thinned using FIB after mechanical pre-thinning of the sample to a thickness around 30 µm

6.2 Operating Mode

6.2.1 Equipment and Supplies

The FIB instrument (focused ion beam) is composed of an ionic column (Fig. 3.35) mounted inside a scanning electron microscope. Abrasion of the material occurs by scanning the ion beam over the area of interest. The ion beam is also used to make either an electronic or ionic image (depending on the detectors available) in order to check the thinning process. The latter procedure is monitored in situ via the electron microscope.

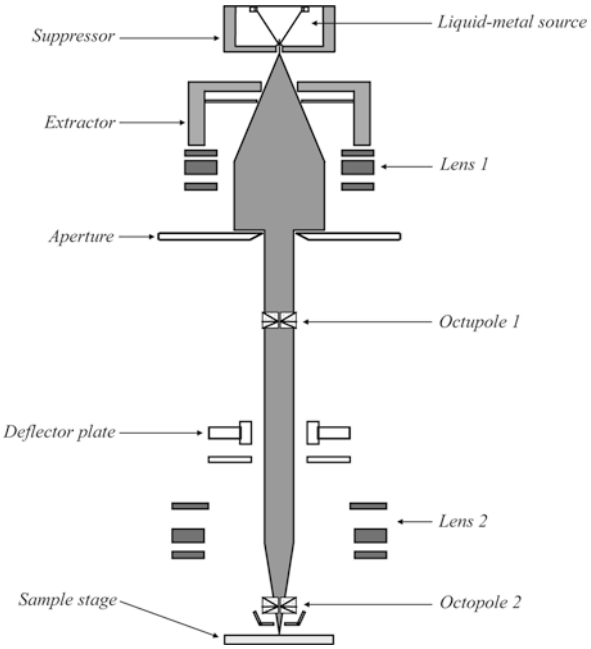


Fig. 3.35 Layout of an ionic column

FIB devices generally work with gallium ions at energies between 1.5 and 30 kV. The size of the ionic probe can be less than 10 nm. One or more metal evaporation sources (platinum, tungsten, etc.), as well as carbon sources, are included in the equipment (Fig. 3.36) to deposit a film that protects the surface of the sample to be milled.

6.2.2 Procedure

Coating the Sample with a Metallic or Carbon Film

After selecting the area of interest in the FIB, it is essential to deposit a carbon or metallic film (platinum, tungsten, etc.), approximately 1-μm thick, on its surface, to protect it during thinning (Fig. 3.37). Metal deposition occurs by chemical

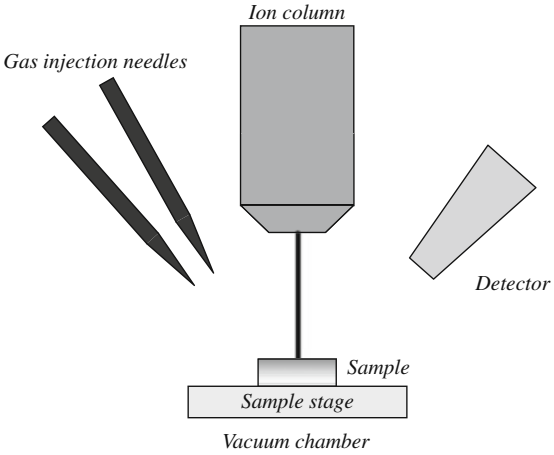


Fig. 3.36 Layout of the positioning of the ion beam, metal sources, and a secondary electron detector for forming the image

vaporization of an organometallic precursor gas. A pre-determined quantity of gas is introduced into the chamber by the opening of a valve and is directed to a capillary situated approximately 100 μm above the sample surface. The gas molecules are adsorbed by the sample surface and decompose only when in contact with the ion (or electron) beam.

In order to prevent ion implantation and surface abrasion by the ion beam during the first moments of metal deposition, a few tens of nanometers of metal must first be deposited using the electron beam. The voltage and current are on the order of 2–3 keV and approximately 1 nA. This deposition is much slower than ionic deposition, and this is why it is used to deposit the first layers only.

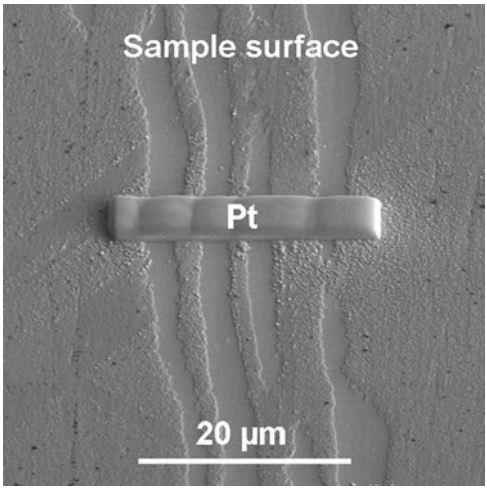


Fig. 3.37 Platinum deposited on the surface of the area of interest. SEM secondary electrons image (*F. Bobard, EPFL-CIME, Lausanne*)

Ionic Milling and Internal Extraction of Lamella

Ionic milling is first conducted using an ion beam of 20–30 kV in normal incidence with regard to the sample surface. Two trenches (cuttings) define the exterior faces on both sides of the area of interest (Fig. 3.38), so as to be close enough that they leave only a slice, a few 100-nm thick in the selected area. The width and depth of these trenches (cuttings) are generally 10 μm (Fig. 3.39).

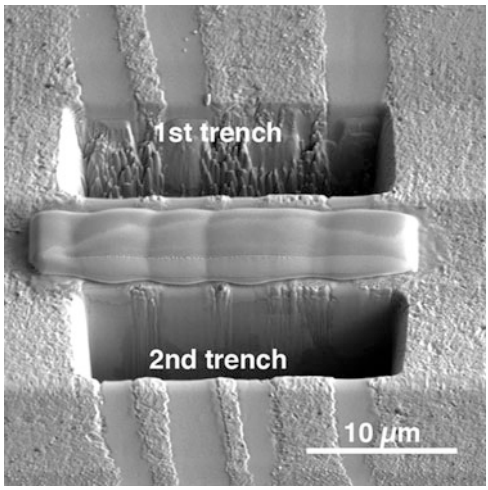


Fig. 3.38 Machining two trenches (cuttings) with high voltage and strong current. SEM image in secondary electrons (*F. Bobard, EPFL-CIME, Lausanne*)

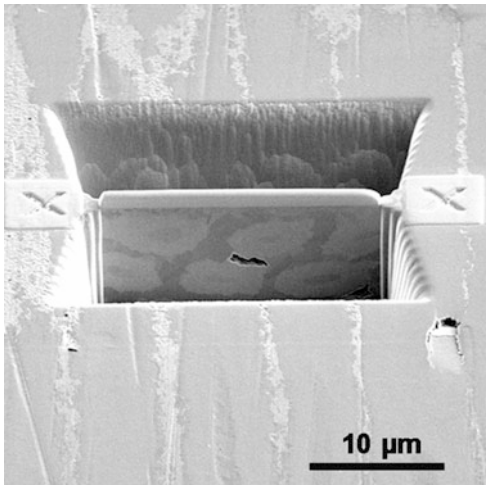


Fig. 3.39 Machining trenches (cuttings) with low voltage and weak current. SEM image in secondary electrons (*F. Bobard, EPFL-CIME, Lausanne*)

The acceleration voltage of the ions is between 1 and 30 keV and current density is on the order of a few tens of picoamps to several hundreds of nanoamperes. The use of high voltage and strong current is necessary to rapidly mill the sample, but this induces significant artifacts (surface roughness, deep ion implantation, amorphization, and temperature rise) which must be minimized or eliminated using low voltage, on the order of 1–2 kV, and a weak current, a few tens of picoamperes.

The slice is then cut using the ion beam, all the way through one side of the slice as well as the base, and only part-way through the other side, in order to provide stability for the following step (Fig. 3.40).

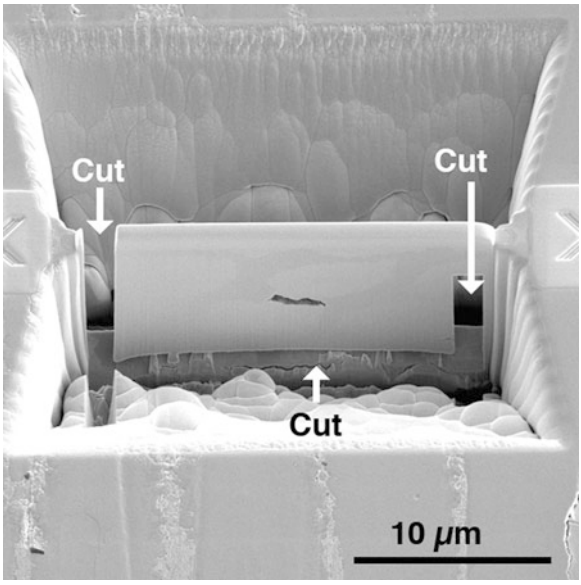


Fig. 3.40 Cutting the slice (*F. Bobard, EPFL-CIME, Lausanne*)

A needle-shaped micromanipulator is moved near the sample. The metal source and the ion beam are used to create a weld between the sample and the micromanipulator. After welding, final cutting of the slice is performed (Fig. 3.41). The slice is then moved (Fig. 3.42) to be welded to a special support adapted to the TEM (Fig. 3.43). The welding method is the same as the previous example.

The following step is to separate the micromanipulator from the slice by ionic abrasion (Fig. 3.44).

Next, the sample is angled from 0.5° to 2° with regard to the ion beam in order to then proceed with final thinning of the pre-thinned area at low voltage (Fig. 3.45). This step is used to repair imperfections and thickness of non-homogeneity of the slice, due to etching with a strong current and the Gaussian shape of the ion beam. It also serves to minimize the thickness of the amorphized layer of the sample surface that is created during machining. The thin slice is removed from the FIB for observation in the electron microscope (Fig. 3.46). It can be thinned again after observation, if necessary.

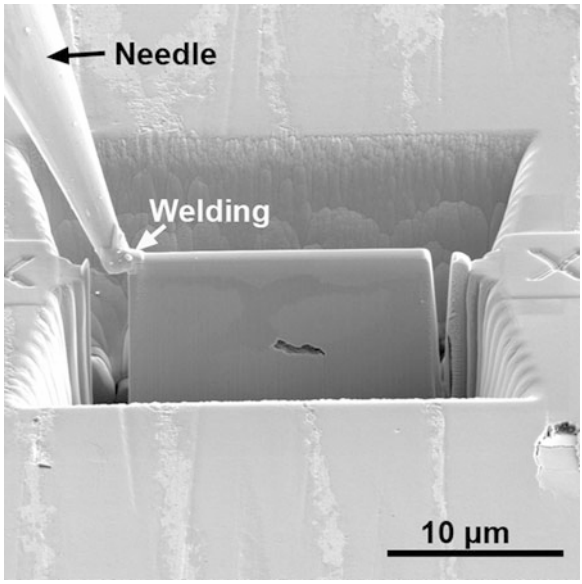


Fig. 3.41 Weld (Pt) between the slice and the needle (micromanipulator) (*F. Bobard, EPFL-CIME, Lausanne*)

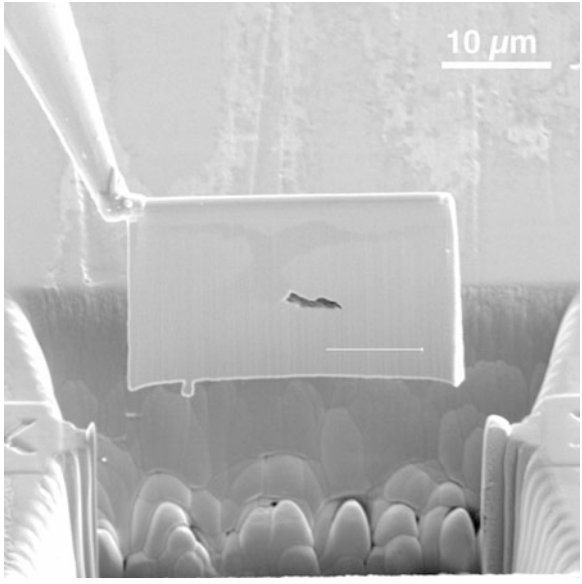


Fig. 3.42 Extracting the thin slice (*F. Bobard, EPFL-CIME, Lausanne*)

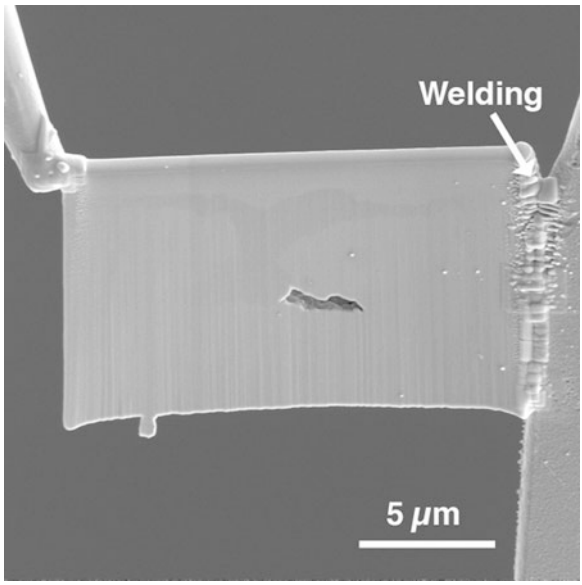


Fig. 3.43 Welding (Pt) the thin slice onto the special support (*F. Bobard, EPFL-CIME, Lausanne*)

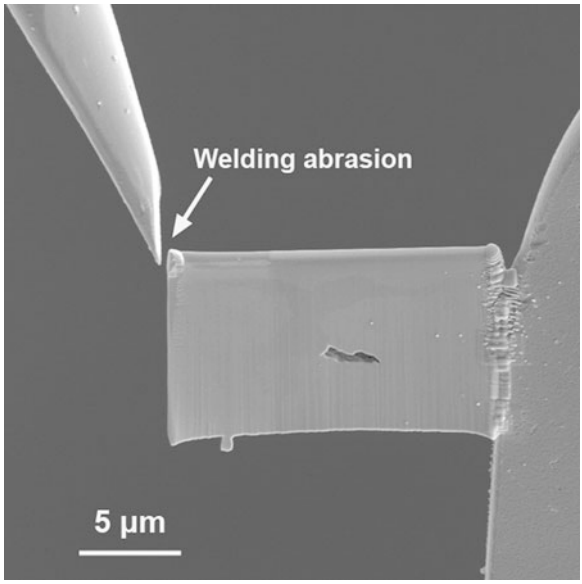


Fig. 3.44 The micromanipulator is separated from the lamella by ionic abrasion of the weld (*F. Bobard, EPFL-CIME, Lausanne*)

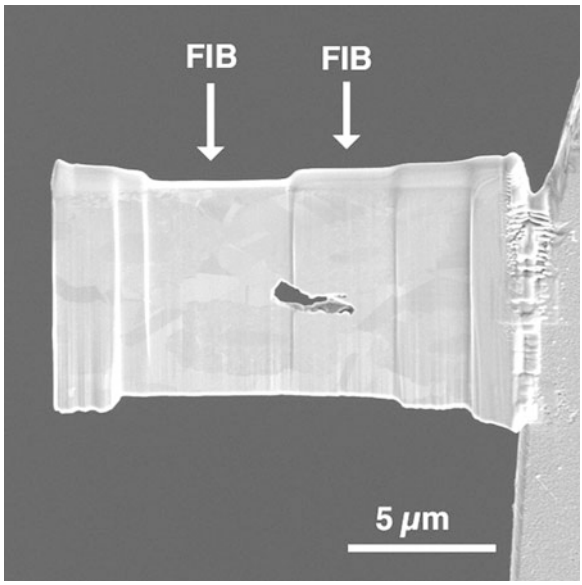


Fig. 3.45 SEM image of an Nb_3Sn multifilament sample, extracted from the bulk material using the internal extraction method. The black contrast in the center of the slice is porosity inherent to the material (*F. Bobard, EPFL-CIME, Lausanne*)

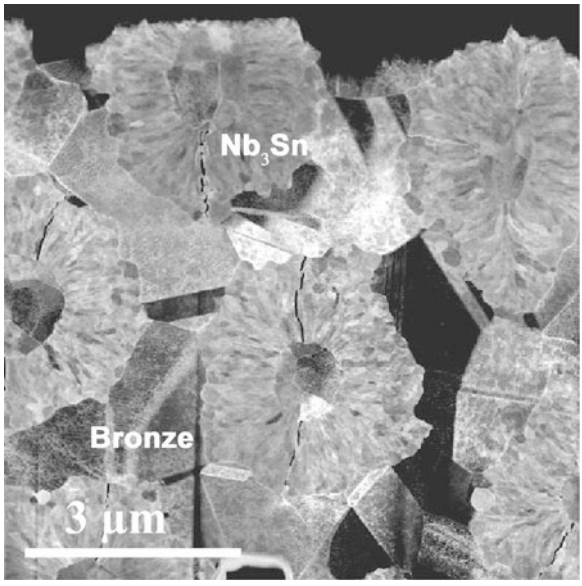


Fig. 3.46 TEM image of an area of an Nb_3Sn multifilament sample. *Note*: There is a homogeneous thinning of the different sample components (*M. Cantoni, EPFL-CIME, Lausanne*)

The thickness of the lamellae is approximately 100 nm. This thickness may be reduced by additional machining at low-voltage and weak-beam current or by chemical etching for some materials.

Several areas of interest can be thinned on the same sample.

Ionic Thinning and External Extraction of the Lamella

High voltage thinning is similar to the process described above. Final thinning, down to a thickness of between 40 and 100 nm, is performed at low voltage, tilting the sample by 0.5° to 2°. Lastly, the sides of the slice, as well as its base, are cut using the ion beam in order to free the lamella.

The sample is delicately removed from the FIB and under an optical microscope; the slice is transferred to a TEM grid coated with a carbon support film, using a fine electrostatically charged glass needle. The thin slice sticks to the needle and can be laid onto the support grid. It can then be observed in the TEM (Fig. 3.47).

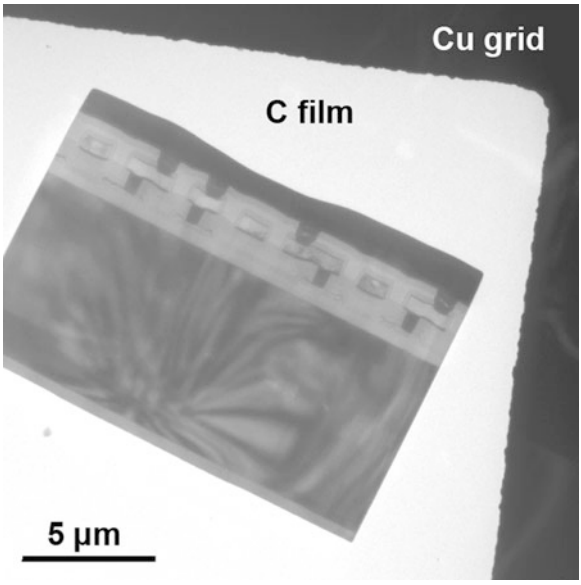


Fig. 3.47 General view of a lamella after the external extraction method. The thin slice is deposited on the carbon film of a coated TEM grid. Bright-field TEM image (*D. Laub. EPFL-CIME, Lausanne*)

Ionic Thinning, H-Bar Method

This method can be used to obtain a thin slice of a brittle material or even an insulating material; in terms of the stability of the thin slice, it is preferable to keep it in place by the neighboring bulk material. The lamella will not be extracted from the bulk.

The sample is cut beforehand to dimensions of approximately 2.5-mm long and 1-mm wide, and then pre-thinned mechanically down to a thickness of approximately 30 µm (Fig. 3.48). A good-quality final polishing facilitates abrasion by the

ion beam. This sample is then stuck to a special support adapted to FIB preparation and to the TEM specimen holder.

A protective metallic layer (Fig. 3.49) is deposited on the surface of the area of interest and ion milling is performed in the same way as in the case of ion milling with external extraction of the thin slice (2) (Figs. 3.50, 3.51, 3.52, and 3.53). The sample can be observed directly in the TEM (Fig. 3.54).

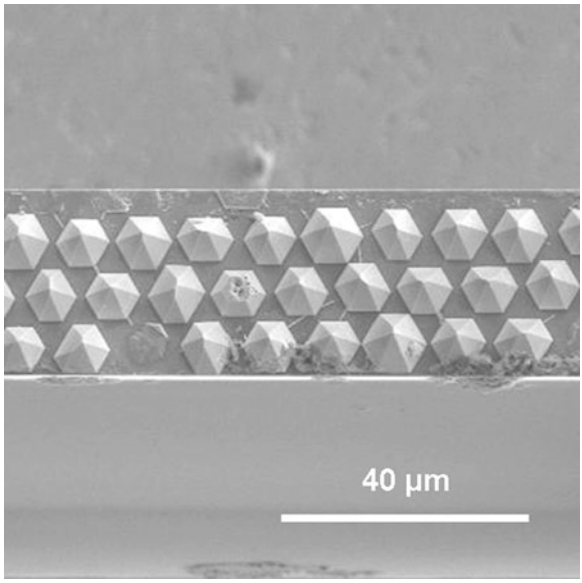


Fig. 3.48 Surface of a sample of GaN pyramids deposited on a sapphire substrate. The sample was mechanically thinned down to 30-µm thick. Secondary electron image, overhead view (*F. Bobard, EPFL-CIME, Lausanne*)

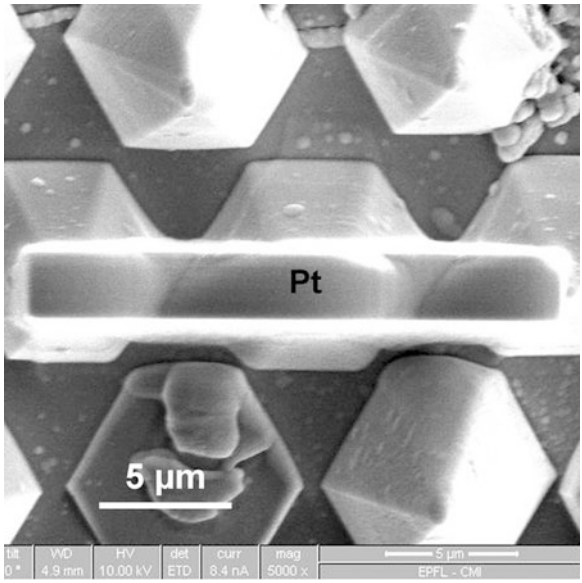


Fig. 3.49 Deposition of a platinum layer on the area of interest. Secondary electron image, overhead view (*F. Bobard, EPFL-CIME, Lausanne*)

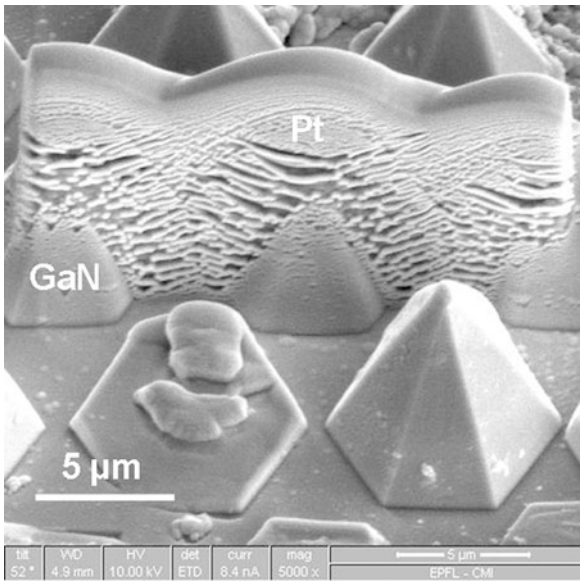


Fig. 3.50 Side view after deposition of the platinum layer. Secondary electron image (*F. Bobard, EPFL-CIME, Lausanne*)

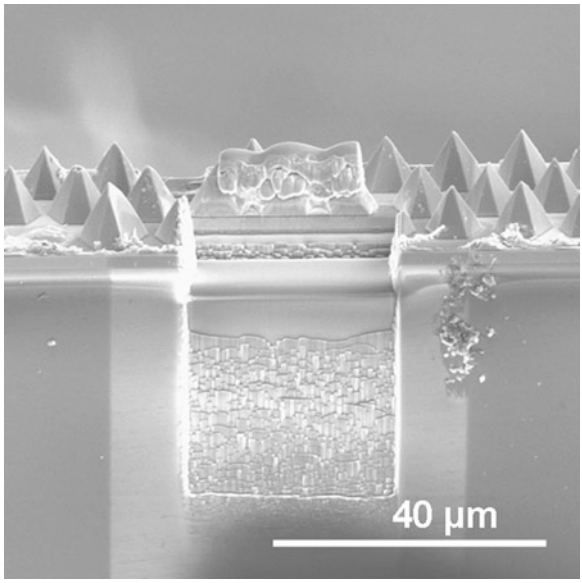


Fig. 3.51 Ionic thinning of the first sidewall, with high voltage and strong current (3 nA). Secondary electron image (*F. Bobard, EPFL-CIME, Lausanne*)

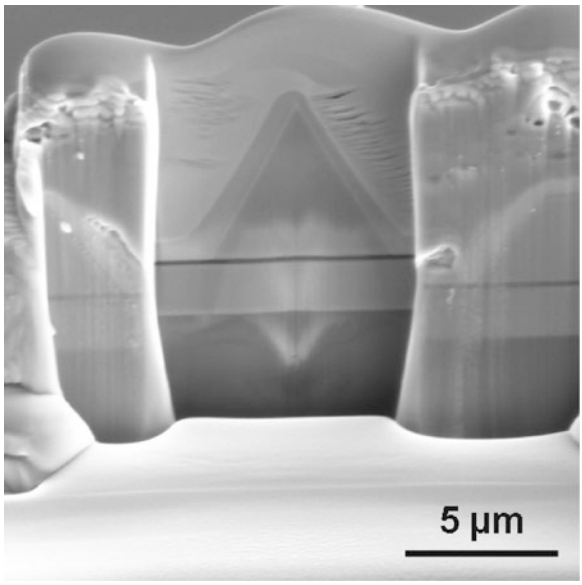


Fig. 3.52 First side after final thinning at low voltage and weak current (30 pA). Secondary electron image (*F. Bobard, EPFL-CIME, Lausanne*)

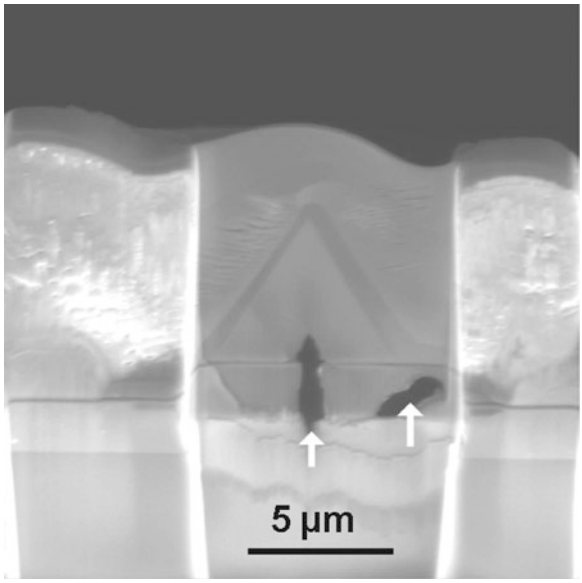


Fig. 3.53 Area of interest after final thinning (30 pA) of the second face. The *arrows* show two holes in the thin slice, either caused by slice bending during thinning or by the effect of redeposition, causing non-homogeneous etching of the slice. Secondary electron image (*F. Bobard, EPFL-CIME, Lausanne*)

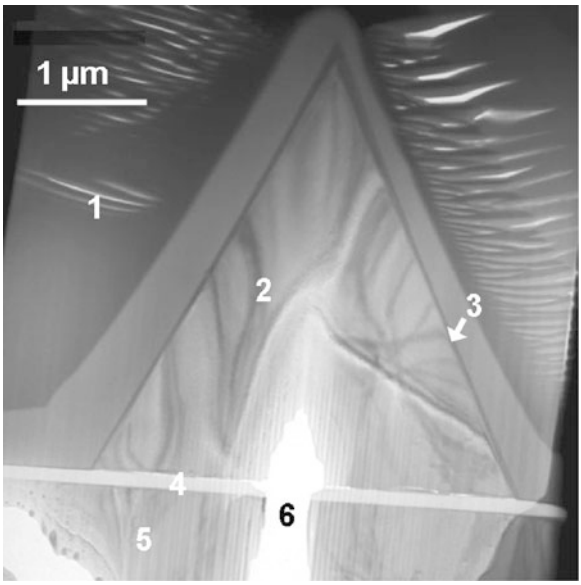


Fig. 3.54 Bright-field TEM image of the lamella: (1) Pt layer; (2) GaN; (3) InGaN; (4) SiO_2; (5) GaN; (6) hole (*C. Tobler, EPFL-CIME, Lausanne*)

6.3 Variants

Cryo-FIB: This is a conventional FIB device equipped with a cold stage. The sample can be cooled beforehand and then cryo-transferred onto the cold stage of the FIB or cooled directly in the FIB. The use of either one of these procedures depends mainly on the nature of the material. The sample can then be thinned, at low accelerating voltage of the ion beam.

It should be noted that FIB is currently used to prepare very soft samples (biological materials and polymers) intended for SEM, i.e., where it is not necessary to produce a lamella. Indeed, making a thin slice as well as extracting it from a very soft sample is not currently possible (with the exception of a few mixed–composite materials).

6.4 Advantages

The area to be thinned is selected with a precision of a few nanometers and can be oriented in any direction. In general, the constant thickness of the lamella makes this an ideal technique for quantitative analysis of chemical composition. Abrasion with an ion beam at an incidence angle between 0° and approximately 2° with regard to the plane of the lamella considerably reduces ion implantation into the material, compared to the ion milling technique. Practically all materials from materials science can be thinned, regardless of their heterogeneity. The secondary electron image generated by the ion beam enables crystallographic contrast by the ion channeling effect (Fig. 3.55b).

A thin slice can also be made from very small samples (from 10 µm).

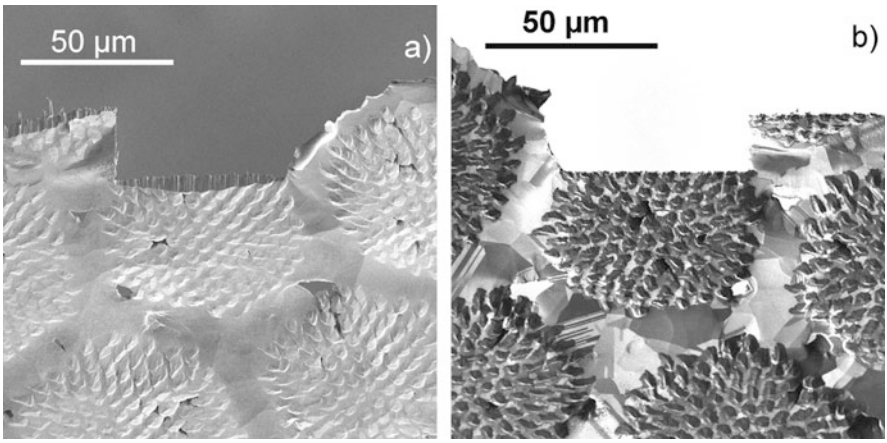


Fig. 3.55 **a** and **b** Comparison between a secondary electron image at 5 kV (**a**) and a secondary ion image at 30 kV, 5 pA (**b**). Ion penetration into the material varies depending on the material and crystallographic orientation. An ion channeling effect appears, depending on the crystallographic orientation of the grains (**b**) (*M. Cantoni, EPFL-CIME, Lausanne*)

6.5 Limitations

Lamella larger than 10 μm^2 are difficult to obtain (lamella deformation). Therefore, the observable area is not very broad. The slice thickness generally does not enable directly performing high-resolution analysis and EELS, which require a thickness less than 50 nm.

A thin slice resulting from FIB preparation on a pre-thinned sample (H-bar method) does not allow for quantitative analysis, because of the proximity of the bulk material.

6.6 Artifacts

The technique can cause structural change. The creation of an amorphous layer on the surface of the lamella induced by ion bombardment can be a hindrance for high-resolution observation. This can be minimized or eliminated by ionic cleaning at low voltage or by a light chemical etching, which is generally for single-phase materials. There is also gallium ion implantation in the sample and redeposition of sputtered matter on the surface of the thin slice. This redeposition can be reduced by a final cleaning at low voltage or by the use of a reactive gas (e.g., Cl_2, I_2, and XeF_2).

6.7 Type of Analysis

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

6.8 Risks

There are no risks.

6.9 Conclusion

FIB is used to prepare multiphase, highly heterogeneous materials which are difficult or even impossible to prepare using other thinning techniques. With ultramicrotomy and cryo-ultramicrotomy, it is the method of choice for EDS chemical analysis and for energy-filtered imaging mode (EFTEM).

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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

J. Ayache et al., *Sample Preparation Handbook for Transmission Electron Microscopy*,
DOI 10.1007/978-1-4419-5975-1_4,

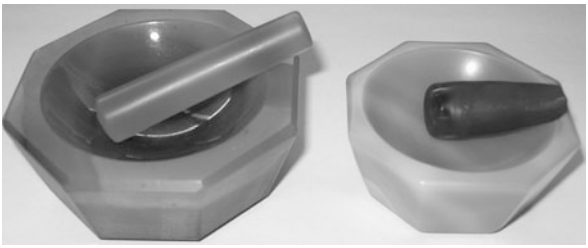

Fig. 4.1 Agate mortars of different sizes. The *darker pestle* is made of corundum (*S. Joulie, IPCMS, Strasbourg*)

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

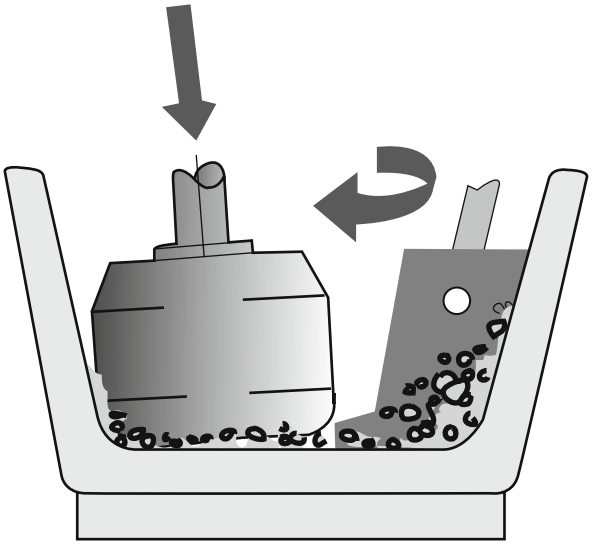

Fig. 4.2 Rotating mortar crusher (*Fisher Bioblock Scientific*)

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.

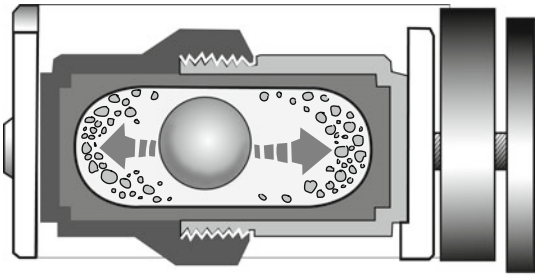

Fig. 4.3 Ball-mill impact crusher (*Fisher Bioblock Scientific*)

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).

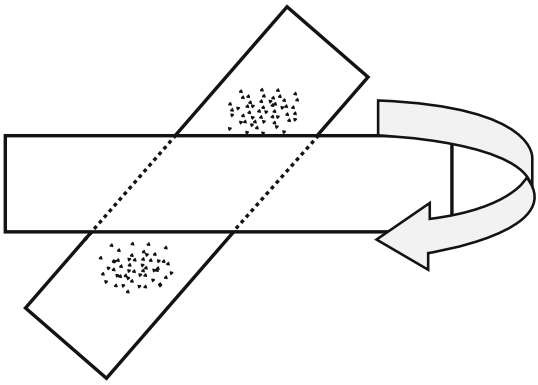

Fig. 4.4 Principle of crushing between two glass slides

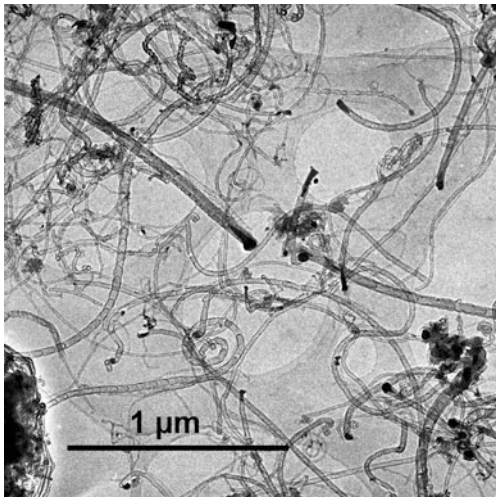

Fig. 4.5 Carbon nanotubes obtained by crushing between two glass slides and recovered dry on a holey film (*C.S. Cojocaru, IPCMS, Strasbourg*)

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).

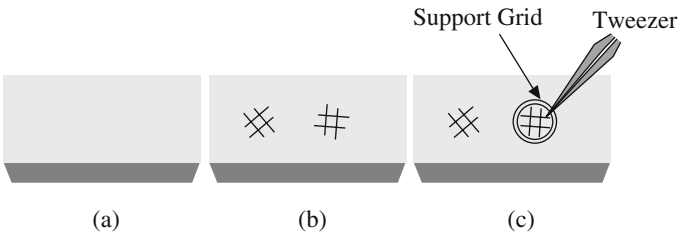

Fig. 4.6 Scraping technique: (**a**) surface to be investigated; (**b**) scratch in the form of a *square* or *pound sign*; and (**c**) sampling on the support grid by lightly rubbing

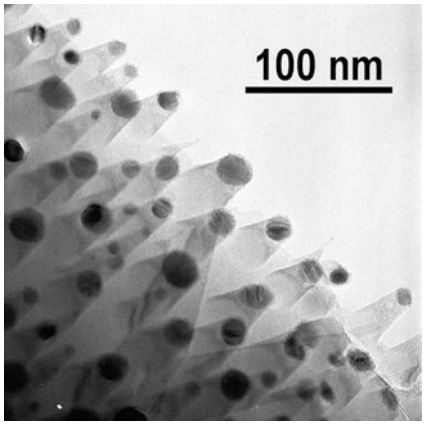

Fig. 4.7 Carbon oxynitride nanopoints obtained by CVD on a single crystal of silicon. Variant of the scraping technique: the material is gathered on a holey film and the orientation of the nanopoints is preserved (*C.S. Cojocaru, IPCMS, Strasbourg*)

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).

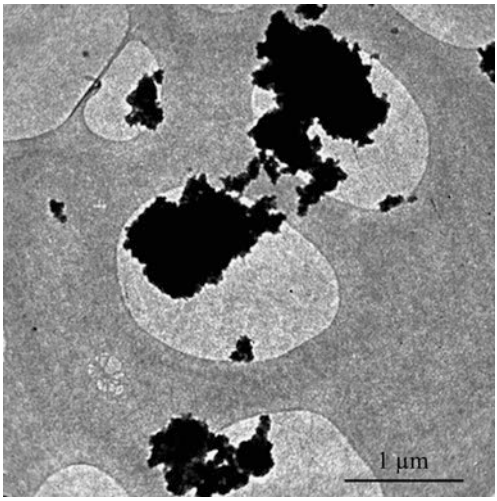

Fig. 4.8 Bright-field TEM image of a catalyst obtained by crushing in an agate mortar, dispersed with ultrasound in distilled water and deposited on a holey film. The material is clumped on the edges of the holes (*G. Ehret, IPCMS, Strasbourg*)

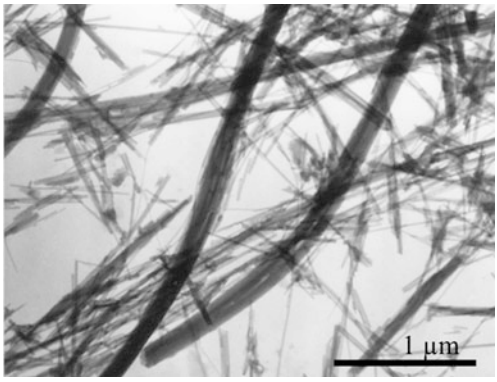

Fig. 4.9 Asbestos fibers (chrysotile) obtained by crushing in an agate mortar, dispersed with ultrasound in distilled water, and deposited on a continuous carbon film. Random fiber orientation (*G. Ehret, IPCMS, Strasbourg*)

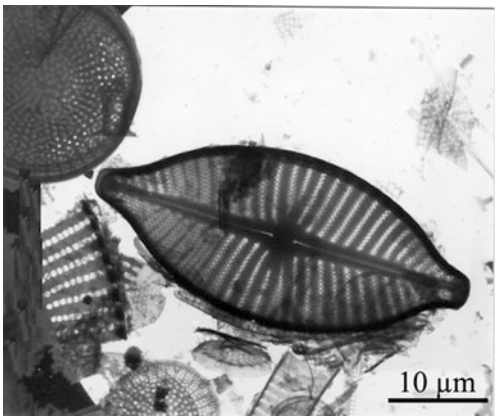

Fig. 4.10 Fossilized diatoms obtained by dry crushing in an agate mortar. Many specimens are broken using this technique (*G. Ehret, IPCMS, Strasbourg*)

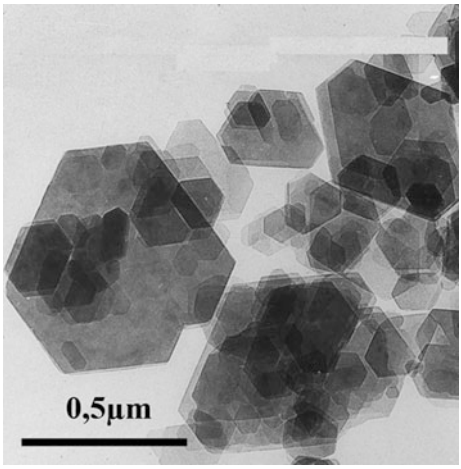

Fig. 4.11 Kaolinite clay particles obtained by crushing in an agate mortar under water and deposited on a continuous film. The platelets (small plates) have a hexagonal facets (*H. Paquet, EOST, Strasbourg*)

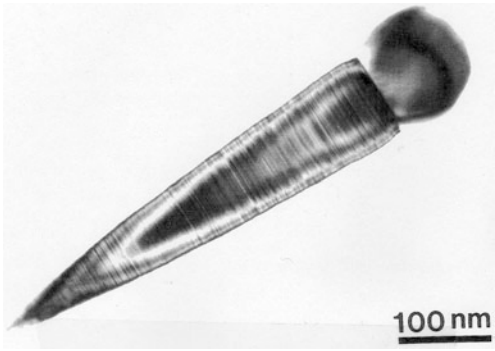

Fig. 4.12 Silicon carbide (SiC) particles in the shape of whiskers. Glass slide variant (*M. Benaïssa, IPCMS, Strasbourg*)

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

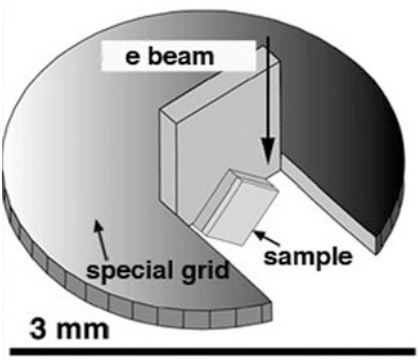

Fig. 4.13 Mounting diagram for the cleaved wedge on the special support disk that makes it possible to orientate the wedge in the TEM

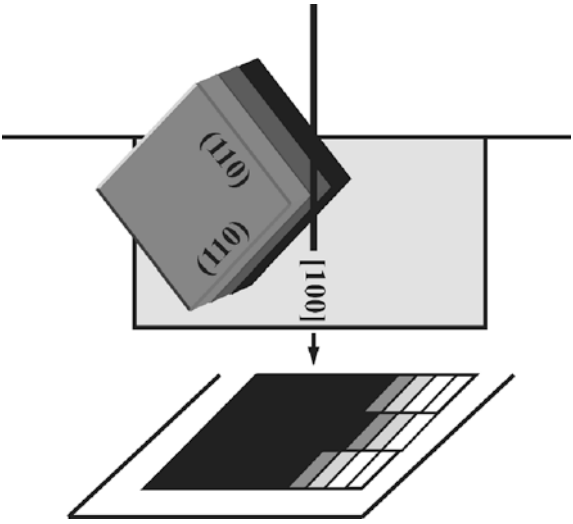

Fig. 4.14 Orientation of the cleaved GaAs wedge for observation in the TEM and the projected image of the wedge

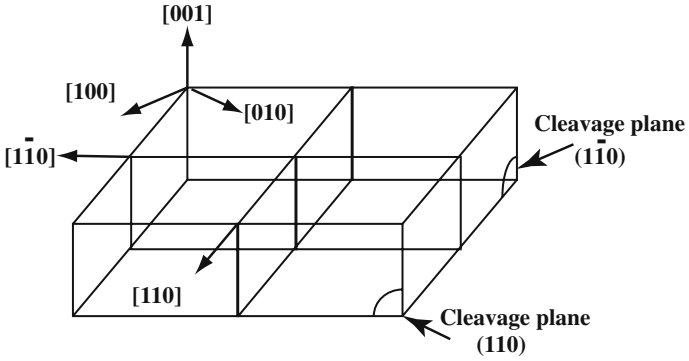

Fig. 4.15 Example of cleavage planes for a GaAs single crystal

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

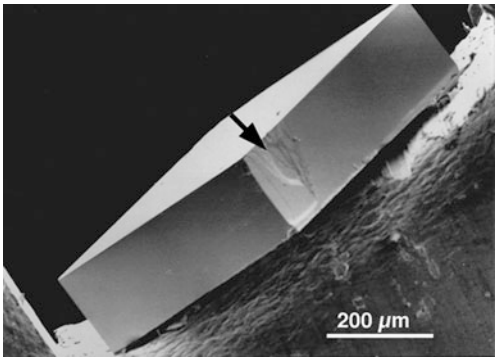

Fig. 4.16 The *black arrow* shows the micro-cracks generated by the initiation of cleavage. Scratching the sample surface with a diamond scriber does cleavage initiation. Secondary-electron SEM image

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.

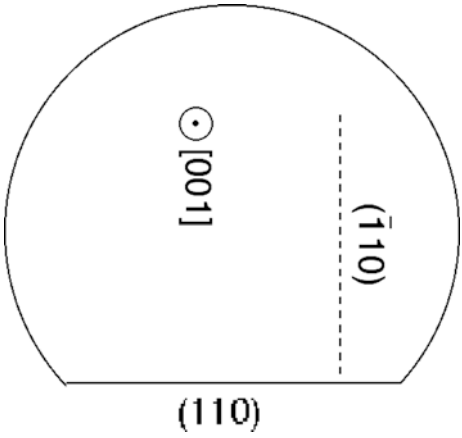

Fig. 4.17 Initial wafer with the indication of the cleavage plane

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.

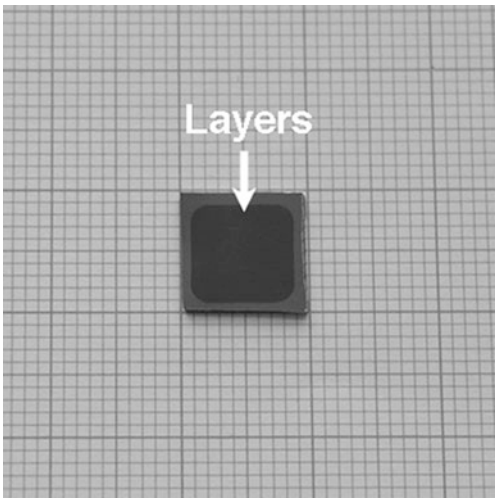

Fig. 4.18 Layers are facing upward

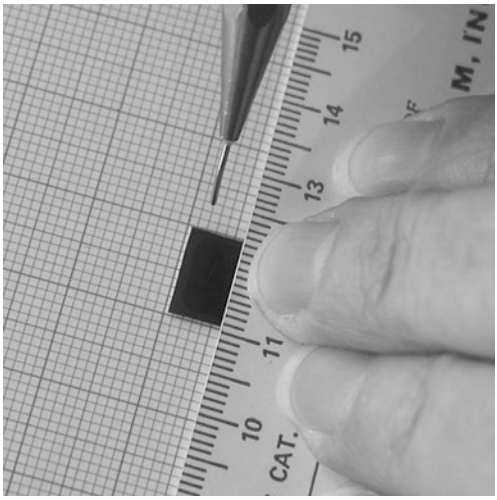

Fig. 4.19 Scoring micro-cracks using a diamond scriber

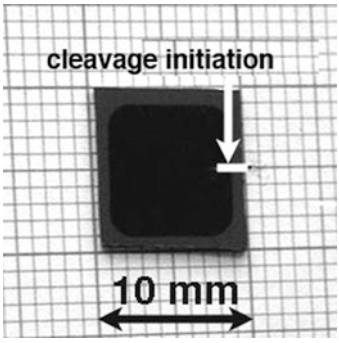

Fig. 4.20 Score mark is 1- to 2-mm long

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).

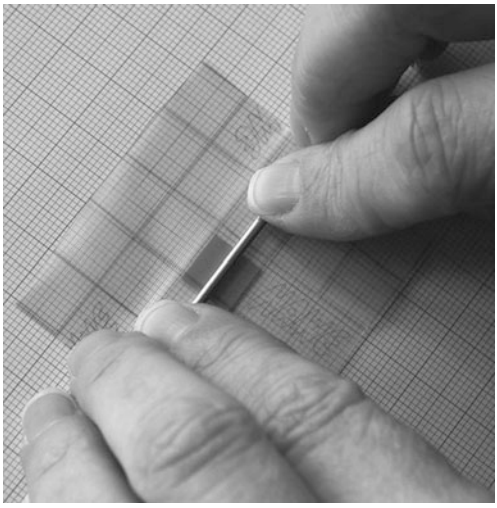

Fig. 4.21 Cleaving the sample using the metal cylinder

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

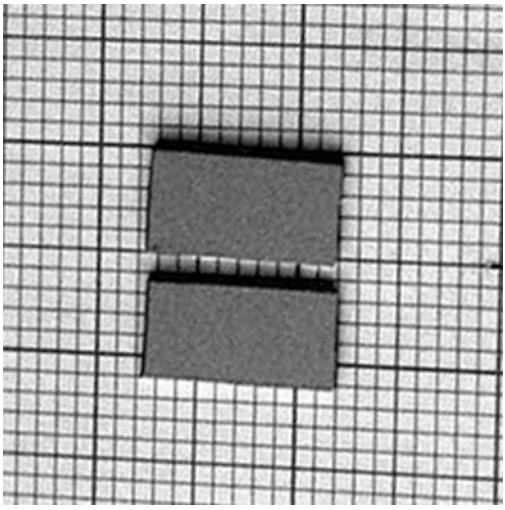

Fig. 4.22 Result after the first cleavage

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.

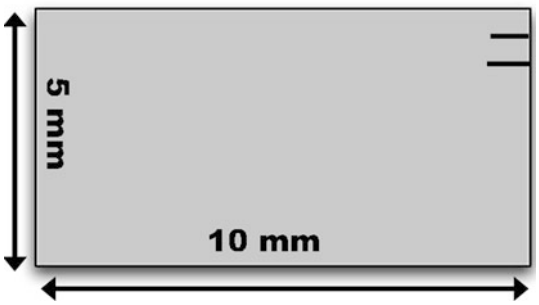

Fig. 4.23 Preparing a 0.5- to 0.7-mm wide strip

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.

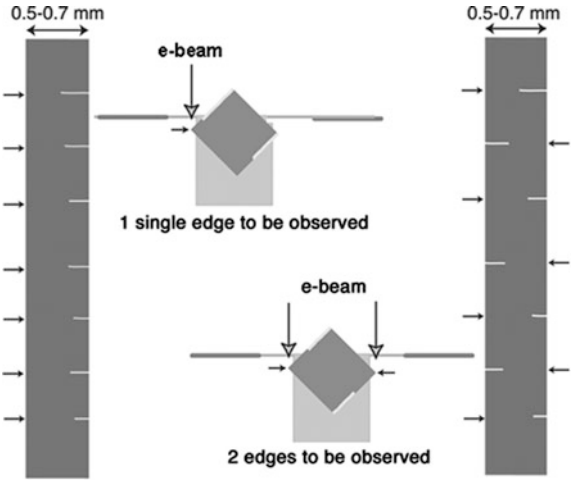

Fig. 4.24 To get two observable wedges on the same sample, the score mark must be made once on the left and once on the right

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.

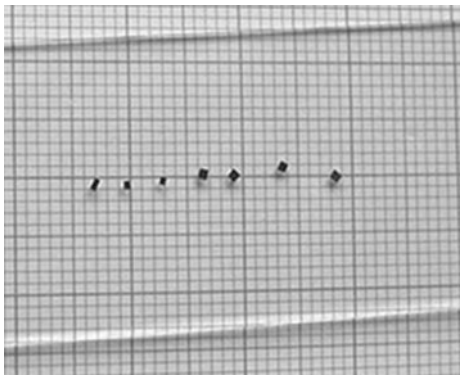

Fig. 4.25 Cleaved wedges are placed on a glass slide, layer-side up, in order to enable selection of the perfect wedge

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).

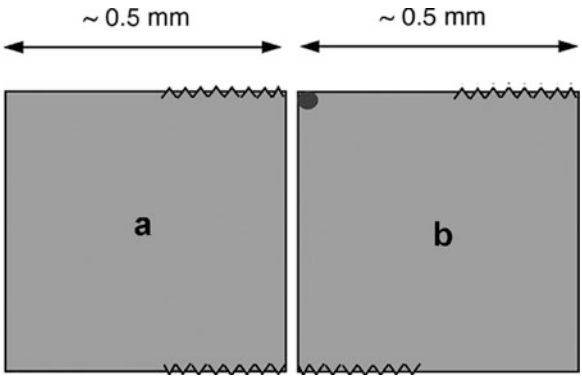

Fig. 4.26 (**a**) Both corners on the *left* are usable; (**b**) the dark spot in the *upper left-hand corner* represents dust deposited on the surface, making the edge unusable. Only the corner at the *bottom right* can be observed

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).

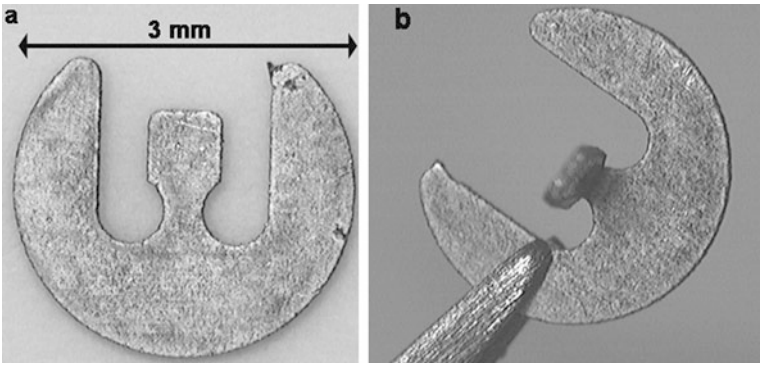

Fig. 4.27 (**a**) A special support cut using electromachining and (**b**) after bending the tab to a 90° angle

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):

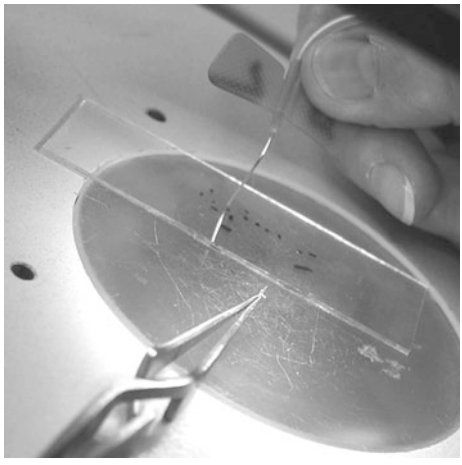

Fig. 4.29 Sample is moved and glued onto the special support

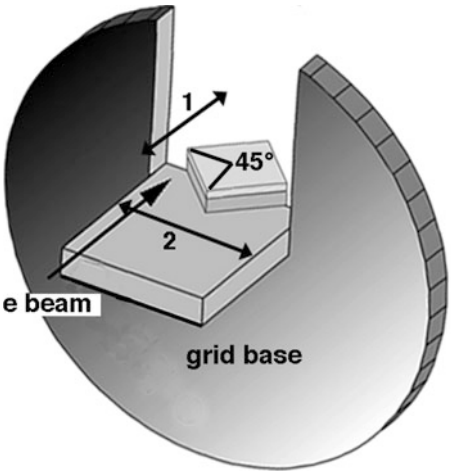

Fig. 4.30 Mounting the wedge onto the support disk. The angle between the incident beam and the edge of the specimen should be 45°

– 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.

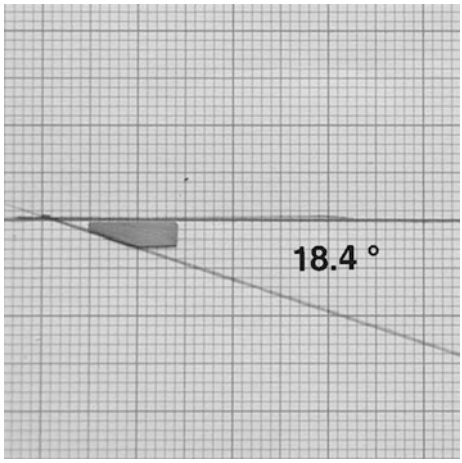

Fig. 4.31 First cleaving at 18.4° of the initial cleavage plane after unilateral mechanical polishing in the cleavage direction

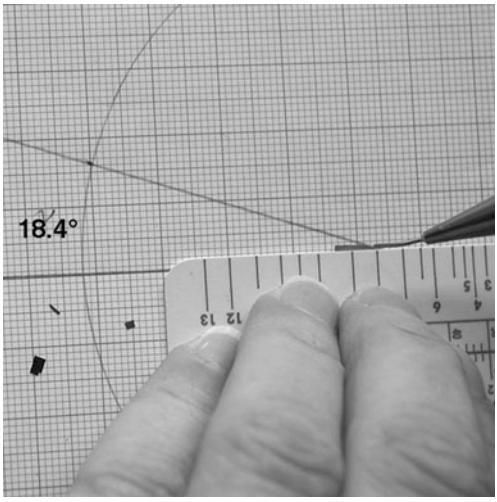

Fig. 4.32 Second cleaving along the initial cleavage plane

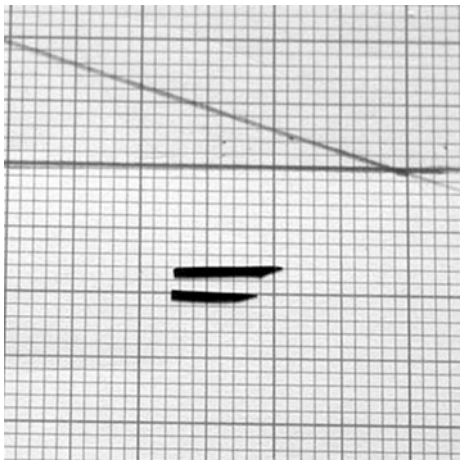

Fig. 4.33 Result after two successive cleavings

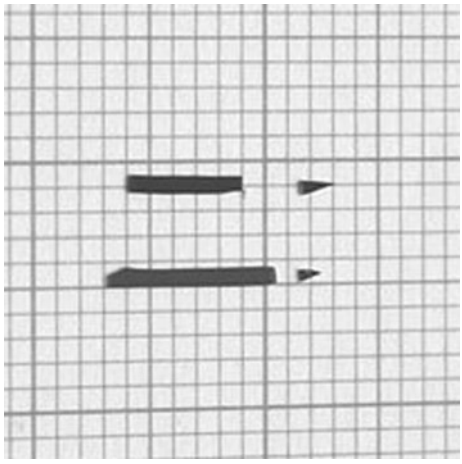

Fig. 4.34 Result after cleaving perpendicular to the initial cleavage plane for producing a small sample

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).

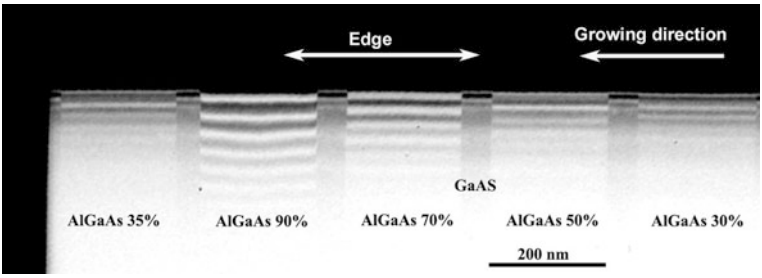

Fig. 4.35 Bright-field TEM image of an AlGaAs/GaAs multilayer. A TEM image combined with a simulation program is used to make a semi-quantitative analysis of the chemical composition from the investigation of equal-thickness fringe contrast. The percentages indicated on the TEM image correspond to the 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

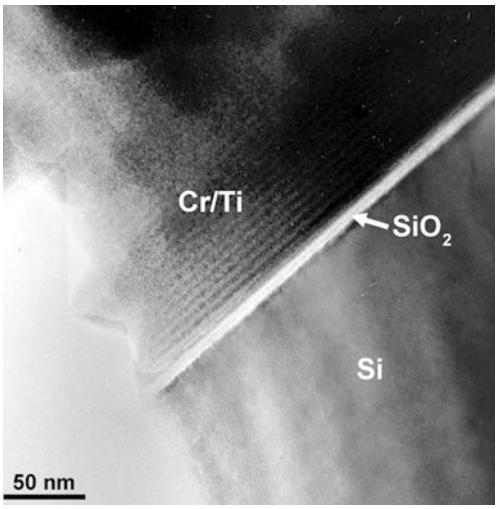

Fig. 4.36 Layers of Cr/Ti-SiO_2 on silicon [111]. The cleaved wedge is used to quickly show the multilayer growth of the Cr/Ti layers near the substrate (*D. Laub, EPFL-CIME, Lausanne*)

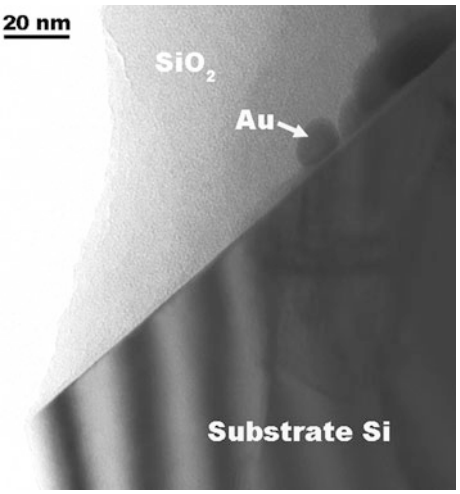

Fig. 4.37 Bright-field TEM image. A SiO_2/Au particle layer on silicon [111] (*S. de Chambrier, A. Schüler, EPFL-LESO-PB, Lausanne*)

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.

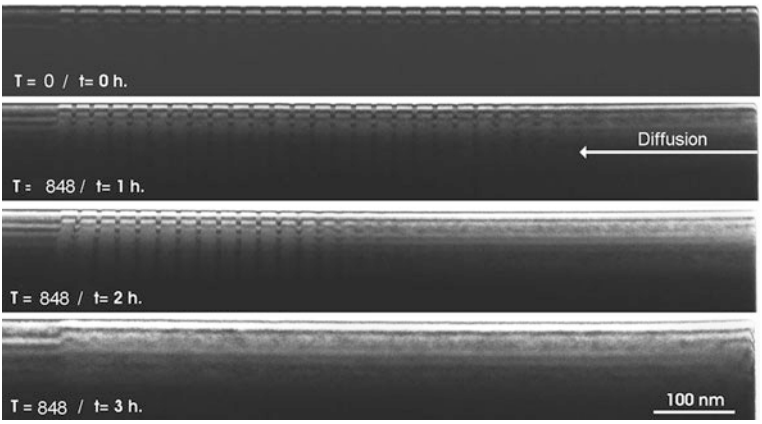

Fig. 4.38 Disorder induced by the diffusion of impurities (Zn) in an AlGaAs semiconductor. Impurity diffusion within a quantum-well structure is accompanied by diffusion of elements from group V (Ga and Al) and consequently can change the chemical composition in a specific area of the structure. The 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).

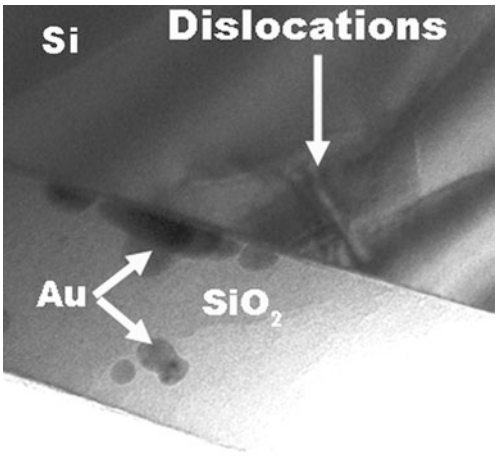

Fig. 4.39 Dislocations in the silicon, probably generated by cleaving (*S. de Chambrier, A. Schüler, EPFL-LESO-PB, Lausanne*)

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).

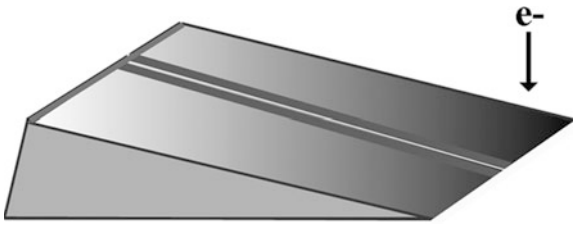

Fig. 4.40 Diagram of a multilayer material "sandwich" prepared for cross-sectional observation. Bevel polishing

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.

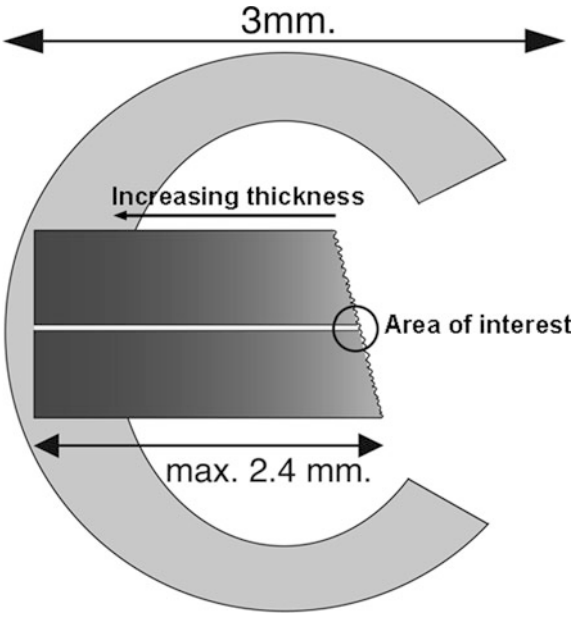

Fig. 4.41 Overhead view of a cross-sectional sample angle polished and mounted on the support washer

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

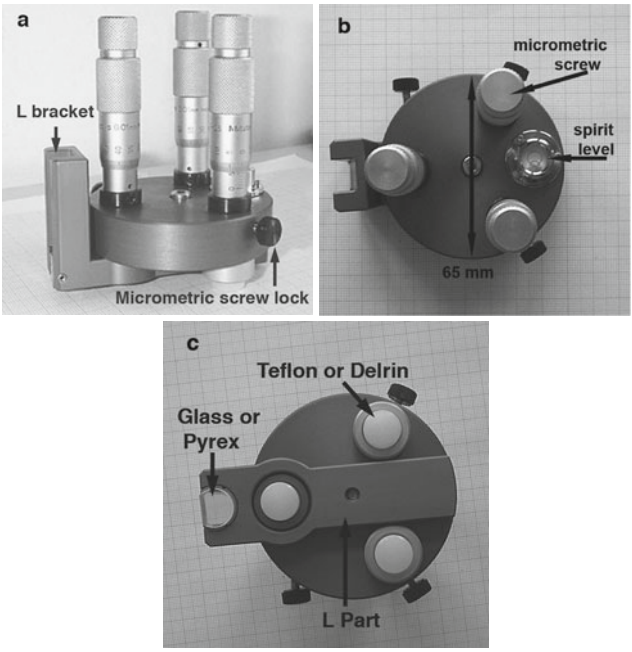

Fig. 4.42 The tripod polisher, seen in (**a**) profile, from (**b**) overhead, and (**c**) underneath

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).

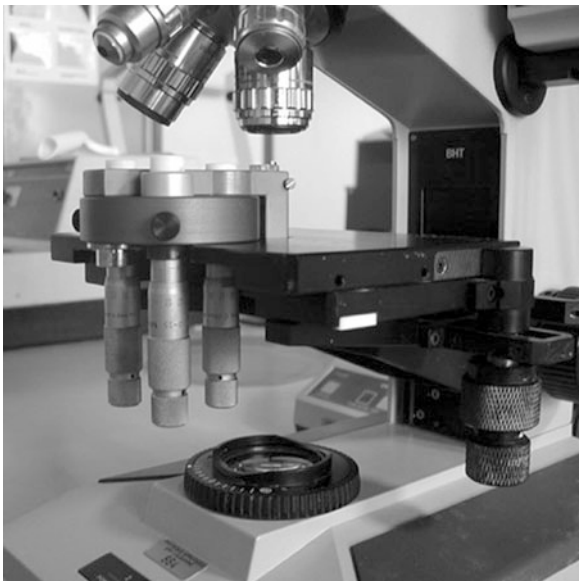

Fig. 4.43 Microscope with modified stage for placing the tripod tool

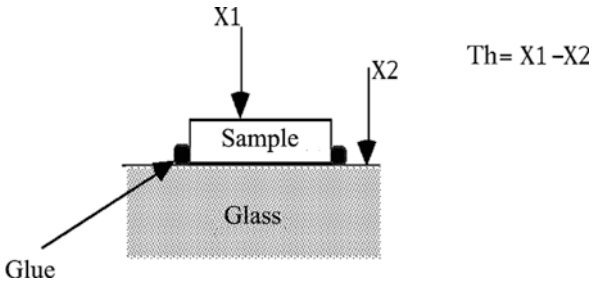

Fig. 4.44 Measuring sample thickness (Th)

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).

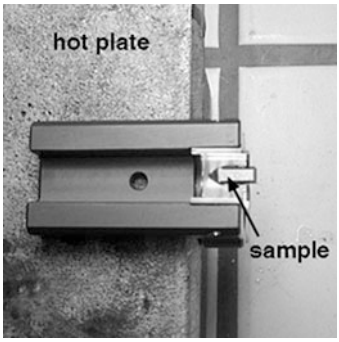

Fig. 4.45 Overhead view

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

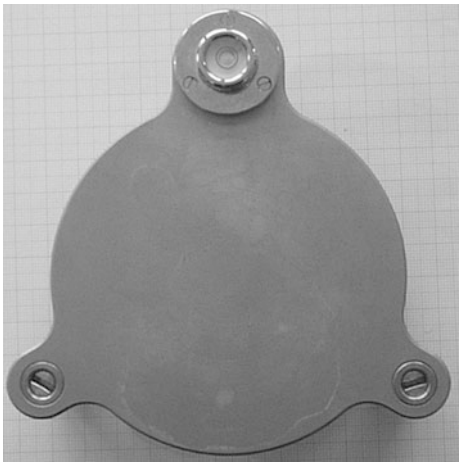

Fig. 4.47 Leveling table

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.

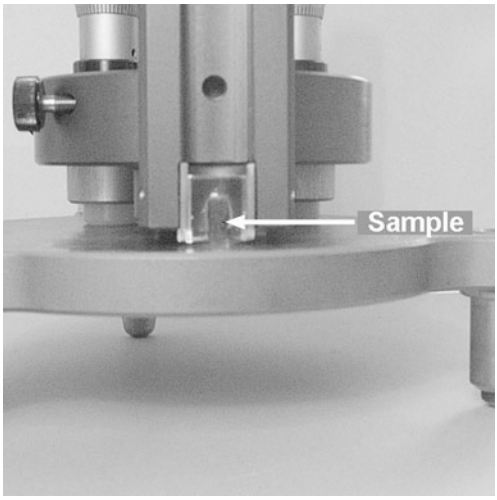

Fig. 4.48 Leveling the rear feet and the sample

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. $1\frac{1}{2}$ 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.

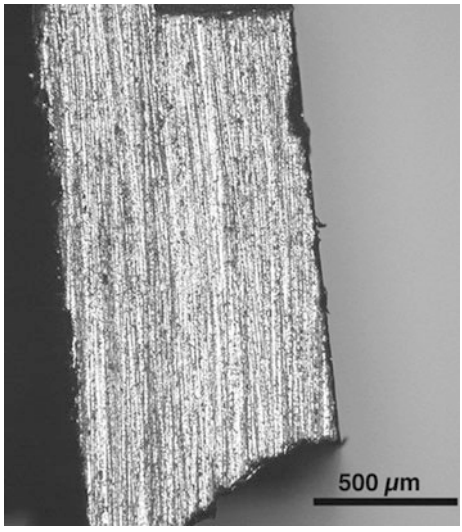

Fig. 4.50 Abrasion with a 15-µm diamond disk

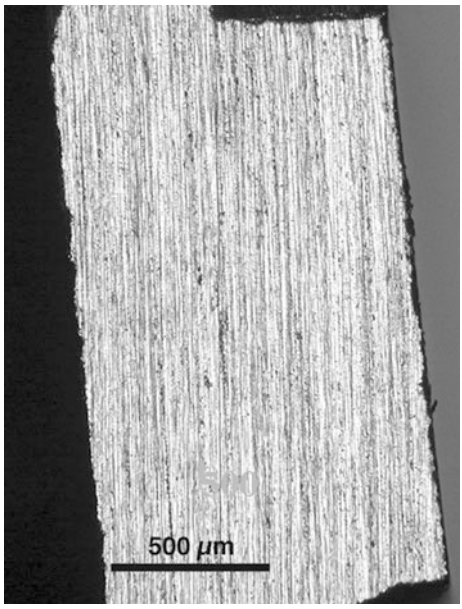

Fig. 4.51 Abrasion with a 6-µm diamond disk

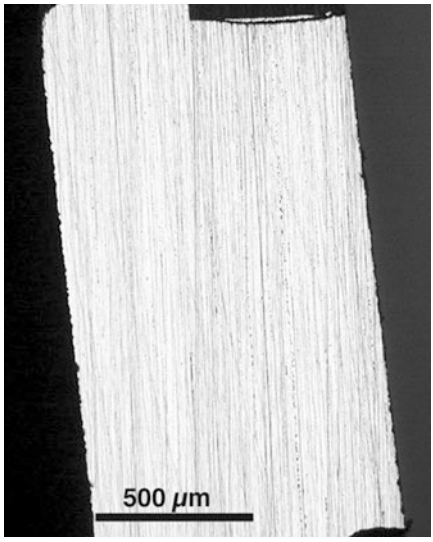

Fig. 4.52 Abrasion with a 1-µm diamond disk. The sandwich glue line becomes visible

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).

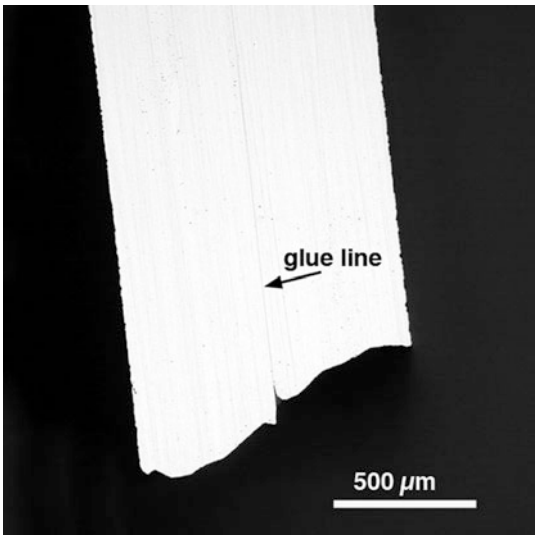

Fig. 4.53 Abrasion with a 0.5-μm diamond disk

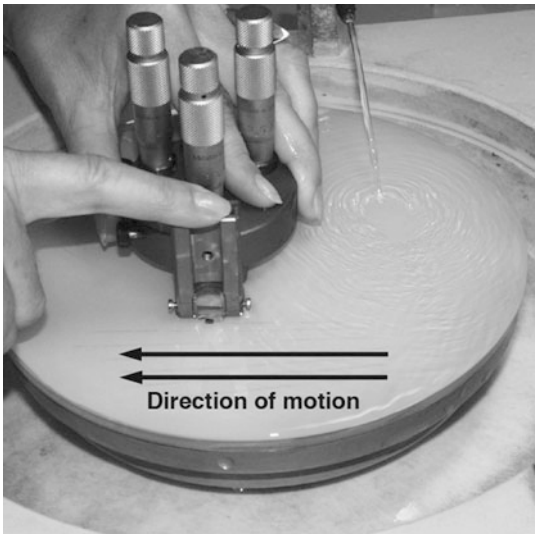

Fig. 4.54 Direction of motion when polishing with a 0.5-μm diamond disk

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.

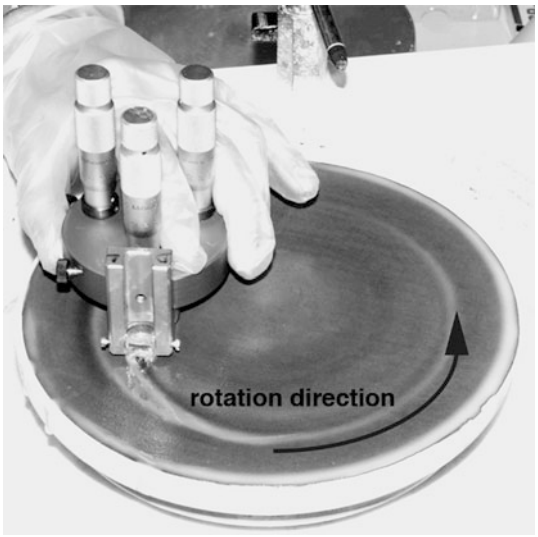

Fig. 4.55 Polishing with the colloid

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.

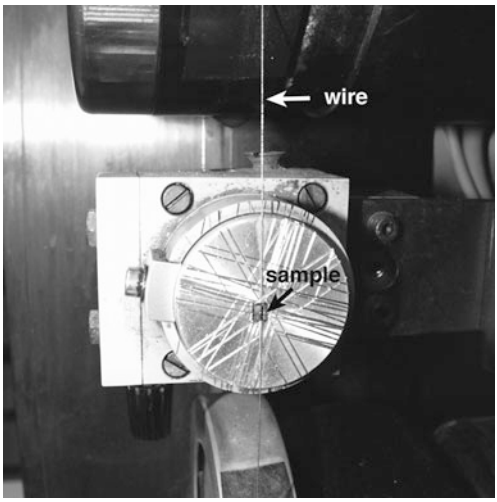

Fig. 4.57 The wire saw enables gentle cutting of the material. The wire diameter, abrasive grain size, and pressure can be selected based on the material brittleness

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.

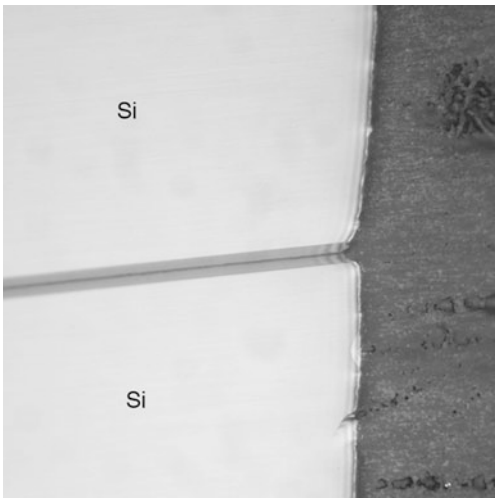

Fig. 4.59 Optical microscopy images, reflected light, of a cross section of a TiO_2/Si sample after final thinning with colloidal silica

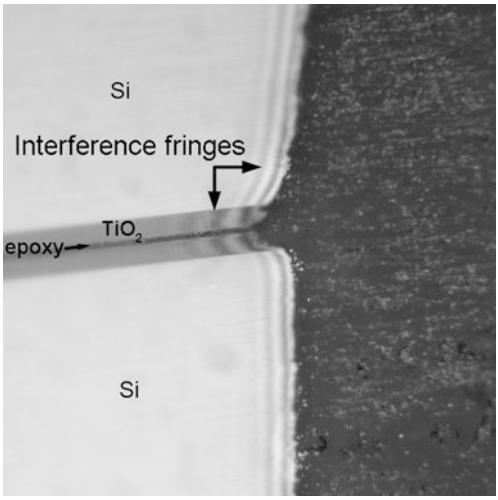

Fig. 4.60 Origin of interference fringes (or equal-thickness fringes): In episcopic illumination (incident light on the observation face), part of the light is reflected by the upper face of the sample and part passes through the sample and is then reflected by the lower face, then exiting the sample 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.

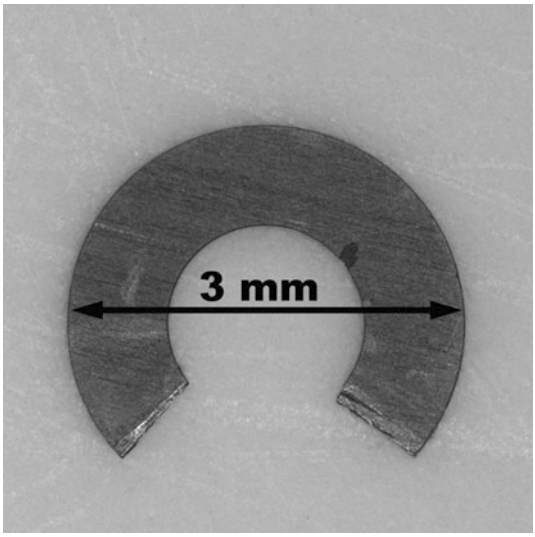

Fig. 4.61 Cut washer. The cutting edges might bend during the operation, so they must be flattened

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.

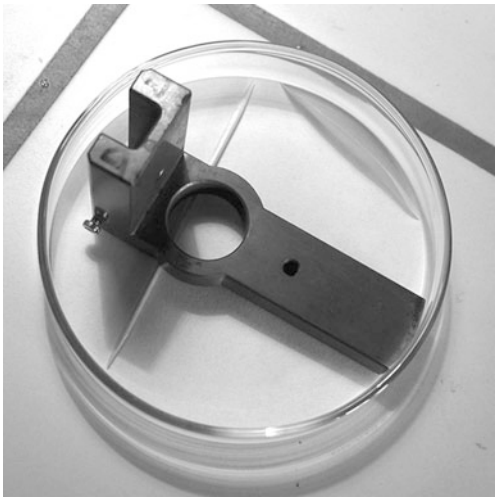

Fig. 4.62 L-bracket with mounted sample, pour in acetone

(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).

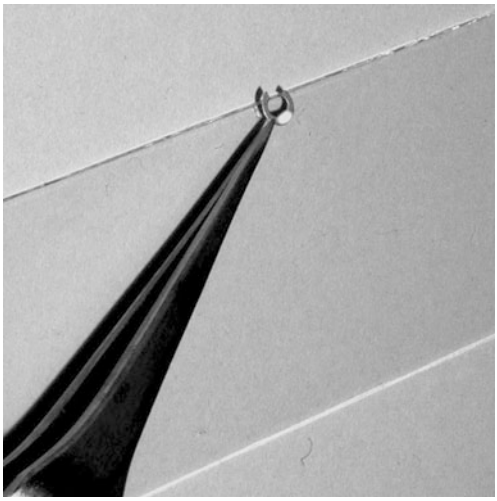

Fig. 4.64 The grid is put on the drop of glue

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

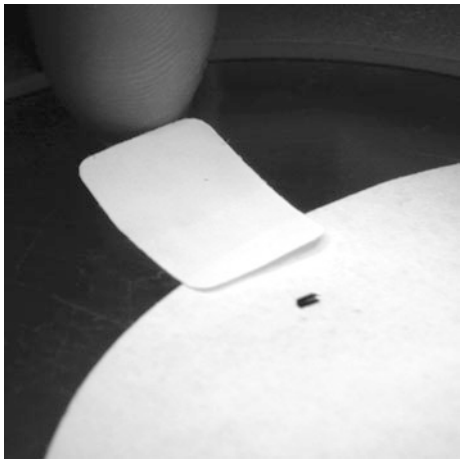

Fig. 4.65 Filter paper is stuck down to prevent moving

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.

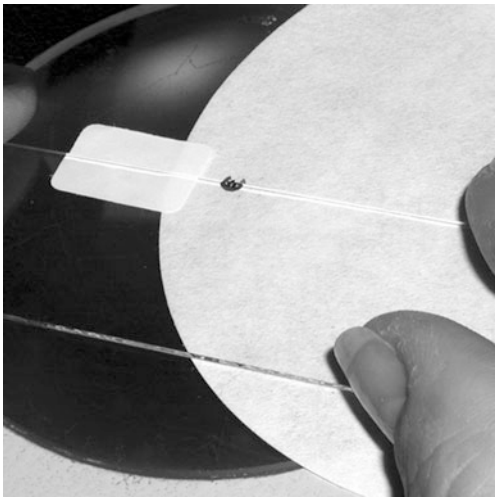

Fig. 4.66 The glass–sample setup is moved until the grid touches the sample

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.

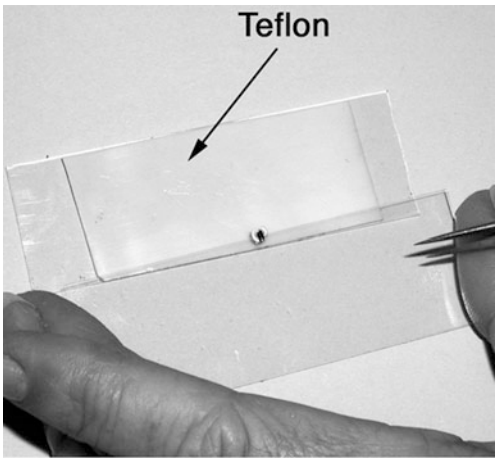

Fig. 4.67 The grid–sample setup is placed on a Teflon plate

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).

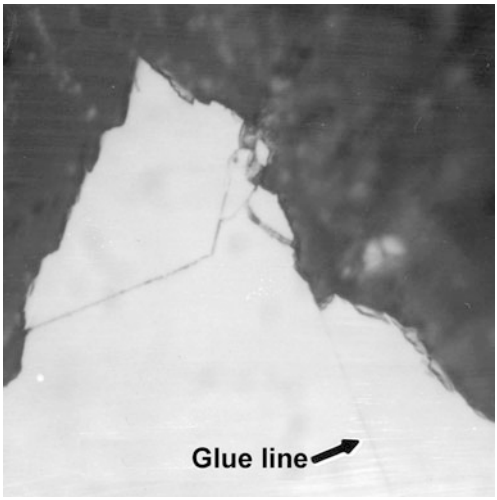

Fig. 4.68 Optical microscope image of a GaAs cross section. The *arrow* indicates the glue line. The sample broke during polishing

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.

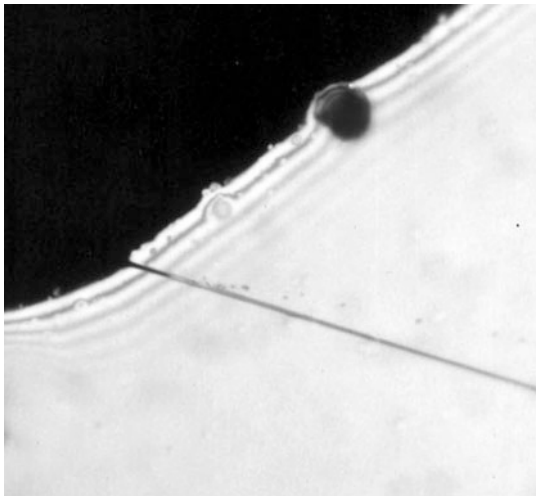

Fig. 4.69 Optical microscope image of the same sample after ion milling with the Gatan Duo-Mill: 1 h at 5 keV, 10 min at 2 keV, incidence angle 16°, two guns, full rotation (experimental conditions)

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.

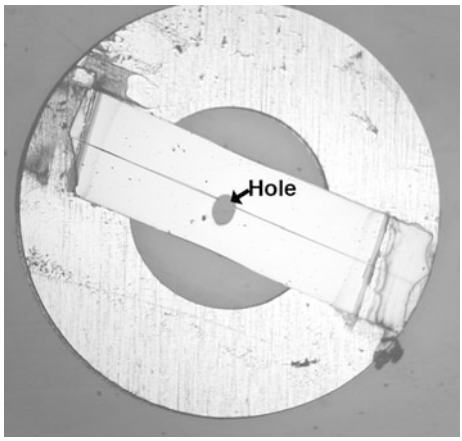

Fig. 4.70 Optical microscope image of a plane polished cross section, after double sectorial rotation ion milling, perpendicular to the sample glue line. The edges of the central hole are electron transparent

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."

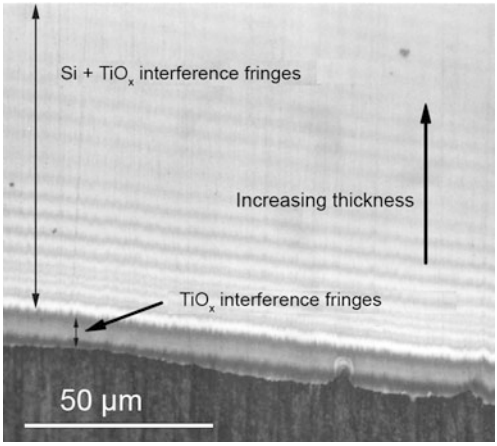

Fig. 4.71 Plane section of a TiO_x/Si [100] multilayer polished to electron transparency. Optical microscope, reflected light

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).

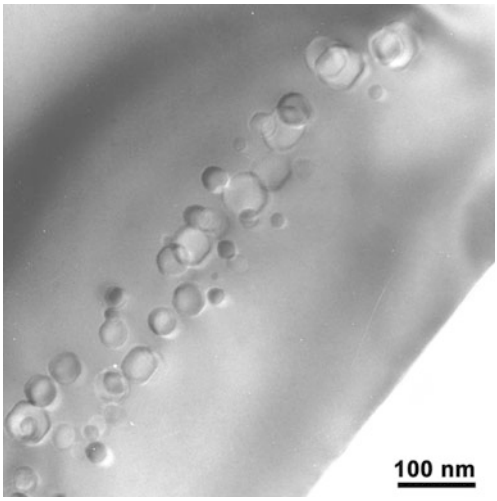

Fig. 4.72 Silicon sample with He implantation (cavities) under the surface. Bright-field TEM image (*J. Werckmann, IPCMS, Strasbourg*)

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.

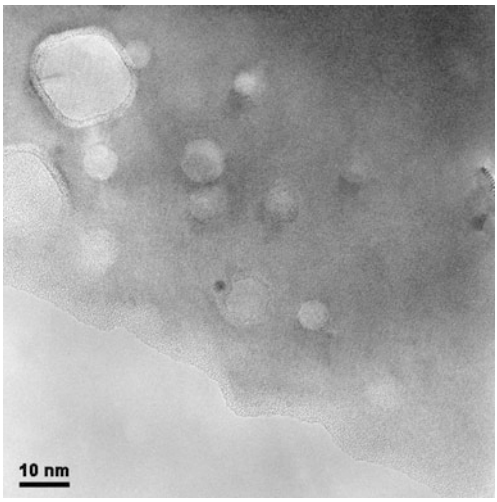

Fig. 4.73 Same sample as Fig. 4.72, thinner area, at higher magnification. Bright-field TEM image (*J. Werckmann, IPCMS, Strasbourg*)

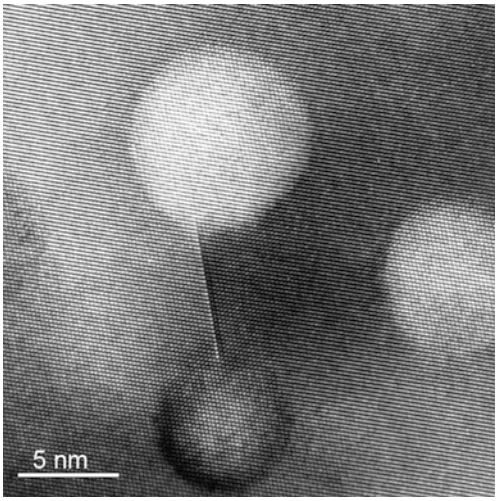

Fig. 4.74 HRTEM image of the same sample showing no surface amorphization (*J. Werckmann, IPCMS, Strasbourg*)

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).

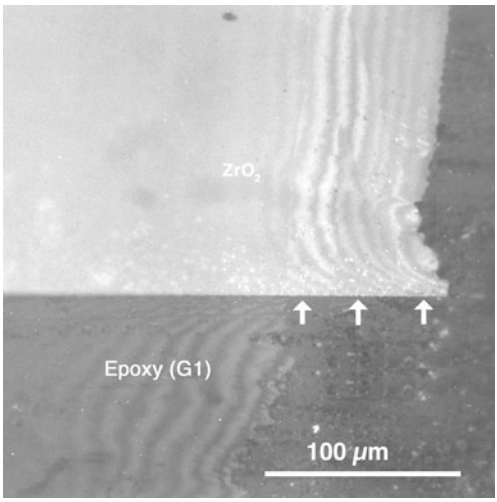

Fig. 4.75 Optical microscopic image of a sample of ZrO_2 in which particles have been implanted on the surface (*white dots*). In this example, the sample was not prepared in a sandwich. A glue film was spread on the surface of the material for cross-sectional wedge preparation. The glue wears away faster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

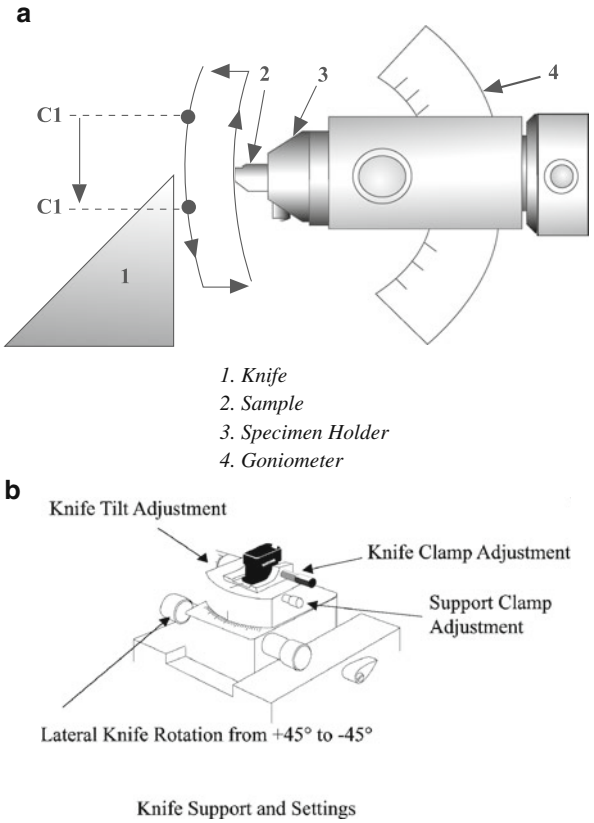

Fig. 4.76 (**a**) Microtome arm/specimen holder assembly and goniometer and (**b**) diagram of the knife settings

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).

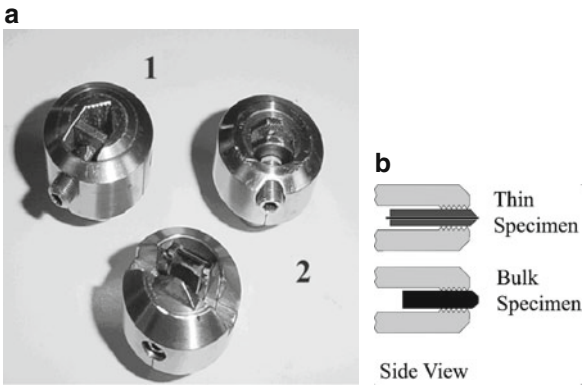

Fig. 4.77 (**a**) Different types of specimen holders: (1) specimen holder designed for a circular or cubic specimen and (2) specimen holders designed for flat specimens. (**b**) There is also a specimen holder for flat samples that does not have any teeth, and the thin specimen is locked between two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

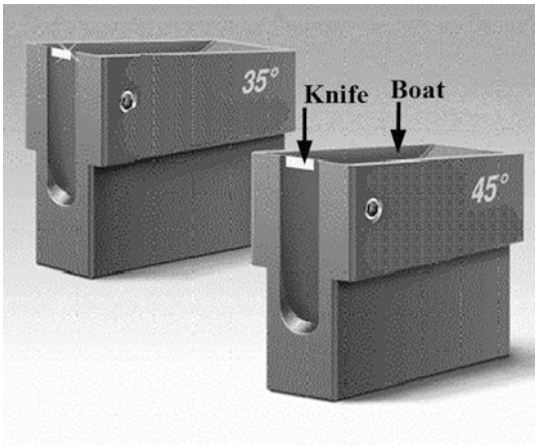

Fig. 4.78 Diamond knives, 35° and 45° angle

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

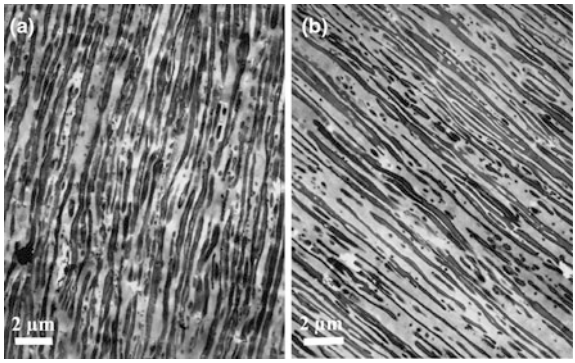

Fig. 4.80 TEM image of a polypropylene/ESI blend (**a**) sectioned at room temperature with a 35° diamond knife without oscillation. The waves are due to compression and (**b**) sectioned at room temperature with a 35° oscillating knife. The sample does not show any sign of compression (*Image courtesy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

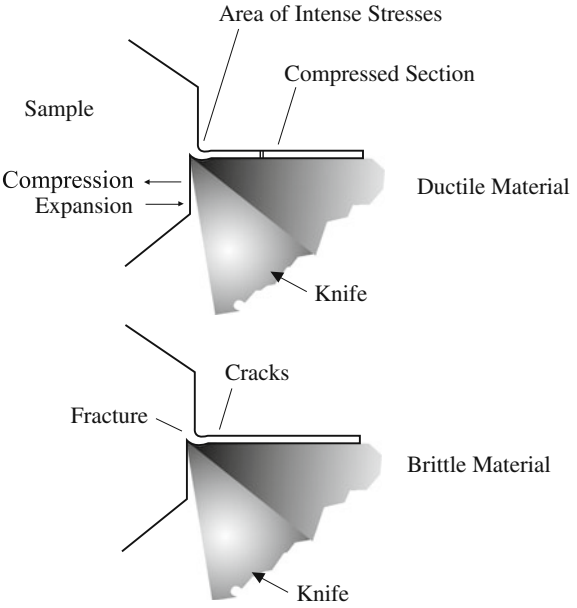

Fig. 4.81 Stresses undergone by a ductile or a brittle material during sectioning

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).

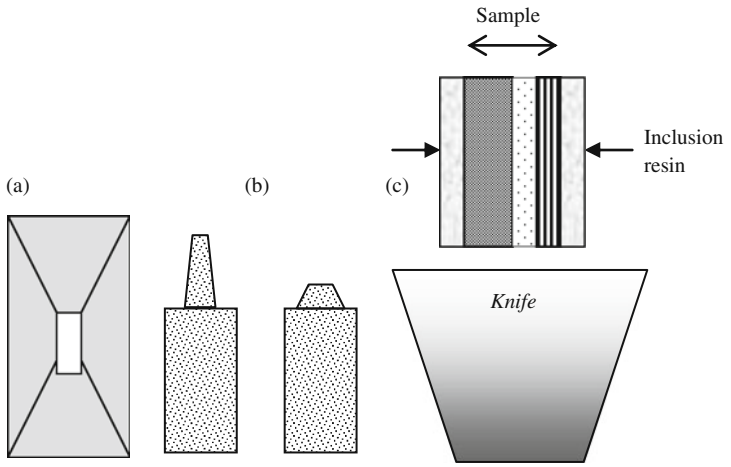

Fig. 4.82 Different views of specimen pyramid

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 × 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

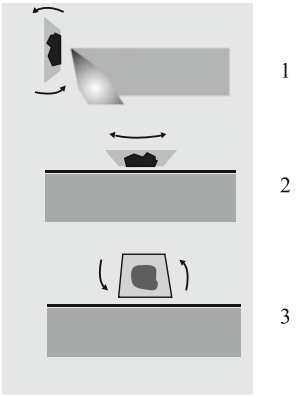

Fig. 4.83 Steps for moving the sample onto the diamond knife: 1, Side view, for judging the verticality of the block with regard to the knife; 2, Front view, for judging the alignment of the block with regard to the knife; and 3, adjusting the alignment

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).

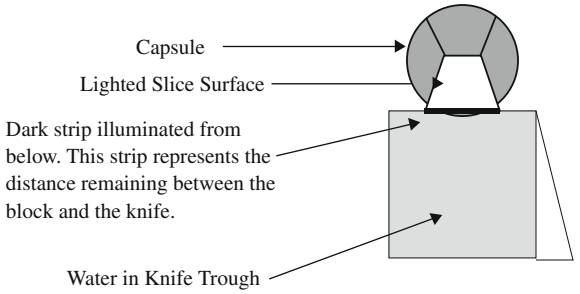

Fig. 4.84 Adjusting the fine approach of the sample block surface and knife

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).

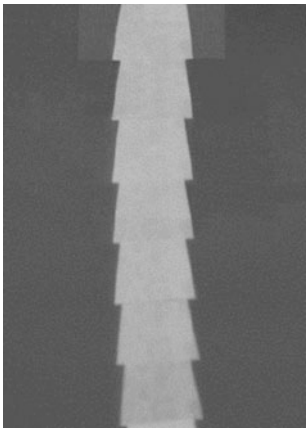

Fig. 4.85 Ribbon of sections on the surface of the water

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).

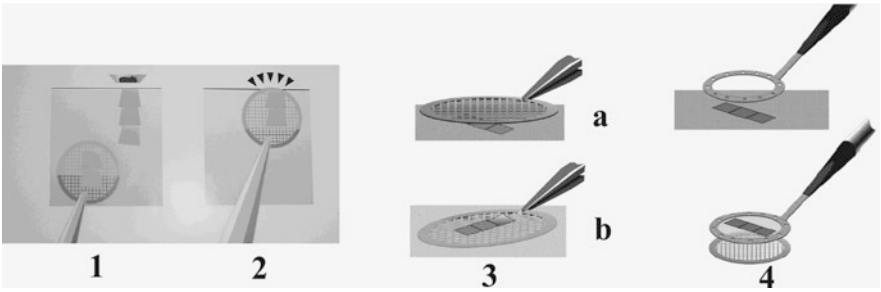

Fig. 4.86 Retrieving sections: 1, proper technique for slices far from the knife edge; 2, too close to the knife edge, risking damage; 3, section retrieval with a grid from above (a) or below (b); and 4, section retrieval with a special "perfect loop" tool

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

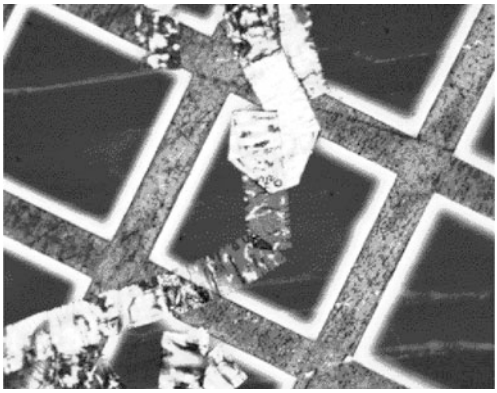

Fig. 4.87 View of a section ribbon deposited on the TEM grid (*H. Gnaegi, Diatome, Bienne, CH*)

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

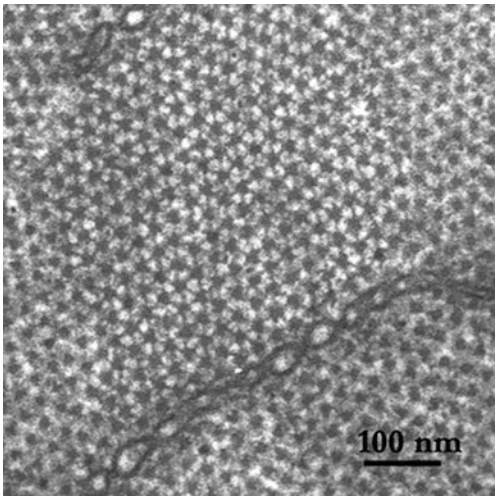

Fig. 4.88 Cross-sectional cut of myosin fibers in skeletal muscle. Chemical fixation, epoxy embedding, cutting, and positive-staining contrast with Ur/Pb (*J. Boumendil, CMEABG UCB-Lyon 1*)

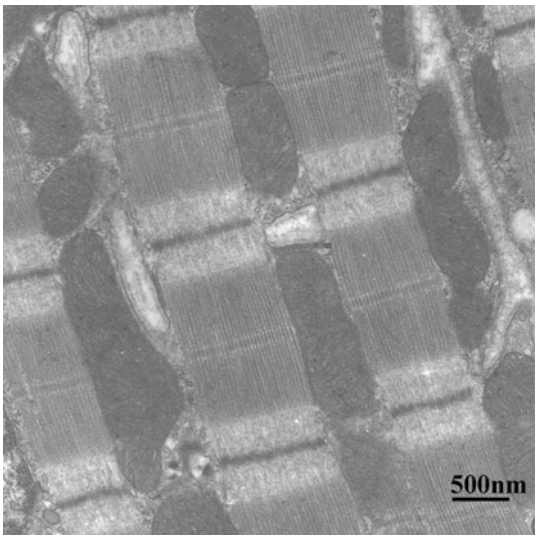

Fig. 4.89 Longitudinal cut of a striated muscle fiber. Alternation of myosin fibrils and actin and mitochondrial cords. Double chemical fixation, epoxy embedding, cutting, and positive-staining contrast (*J. Boumendil, CMEABG UCB-Lyon 1*)

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).

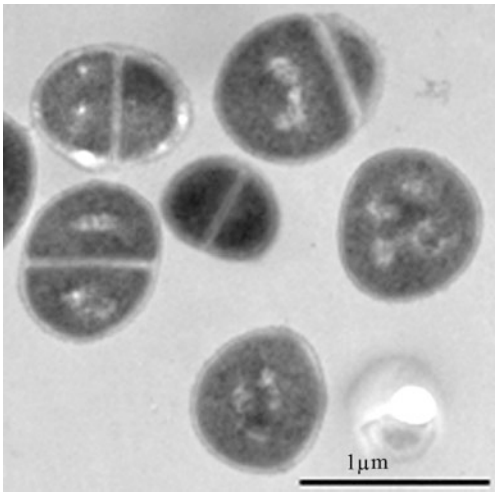

Fig. 4.90 *Staphylococcus aureus* bacteria, with double fixation, embedded in agar, dehydration, embedding in epoxy resin, cutting, and Ur/Pb contrast (*A. Rivoire, EZUS-UCB-Lyon 1*)

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).

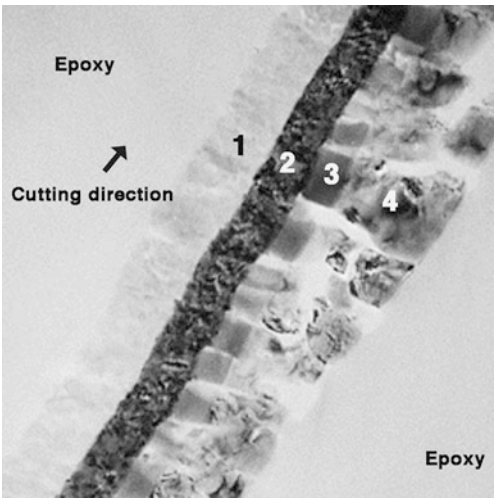

Fig. 4.91 A DCL(1)/Ti(2)/SiO_2(3)/Si(4) alloy multilayer embedded in epoxy resin, showing chatter and fractures in layers (3) and (4) (*H. Gnaegi, Diatome, Bienne*)

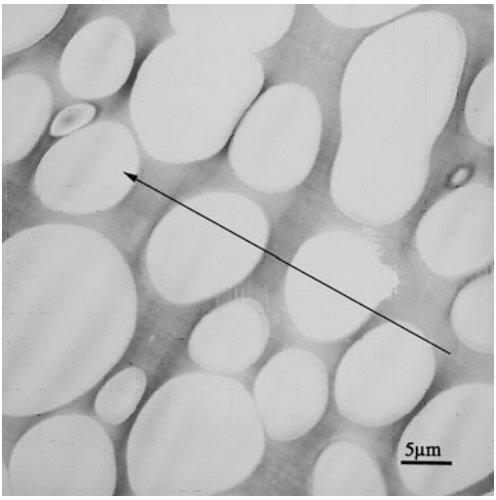

Fig. 4.92 Biphase polymer material. Chatter perpendicular to the cutting direction (*arrow*), resulting from compression (*stresses*), which does not disappear with the surface tension of the water where sections are draped (*A. Rivoire, EZUS-UCB, Lyon 1*)

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.

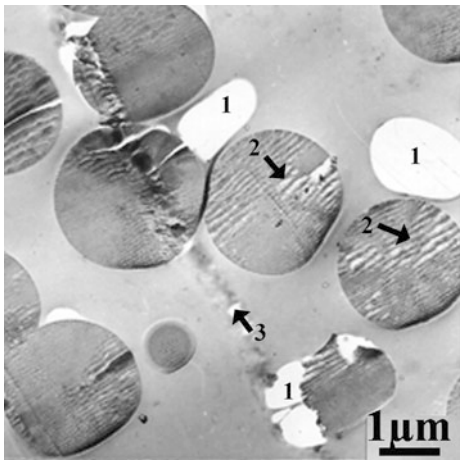

Fig. 4.93 Carbon mesophase particles embedded in epoxy resin showing holes (1), and chatter (2) and knife marks (3) (*J. Ayache, CNRS-UMR 8126- IGR, Villejuif*)

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.)

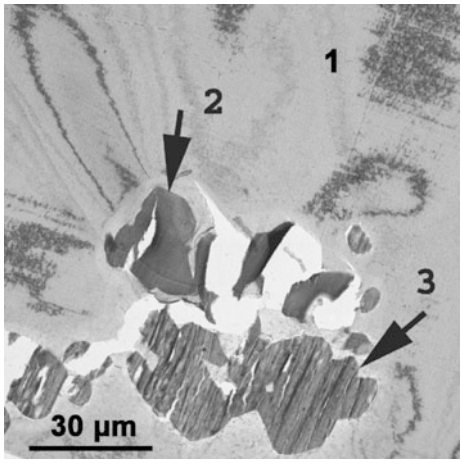

Fig. 4.94 Bulk aluminum-based alloy showing material tears in the section, formed due to mechanical behavior heterogeneity between the precipitates and the matrix. Precipitate 2, which is harder than precipitate 3, is expulsed from the cut. The hardness of precipitate 3 is greater than the matrix, but 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.

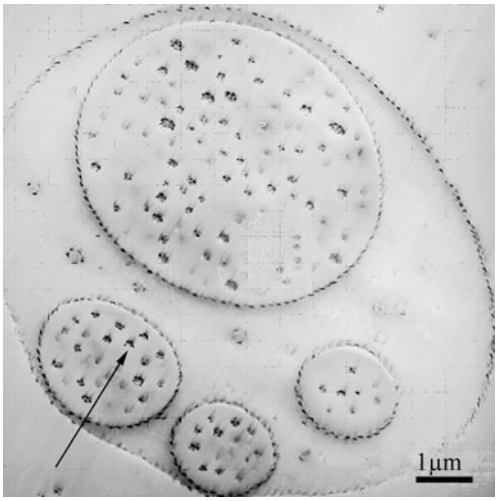

Fig. 4.95 Scratching of some overcharge particles in a polymer matrix. Deformation is in the cutting direction (*arrow*) (*A. Rivoire and E. Girard-Reydet, EZUS-UCB, Lyon 1*)

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).

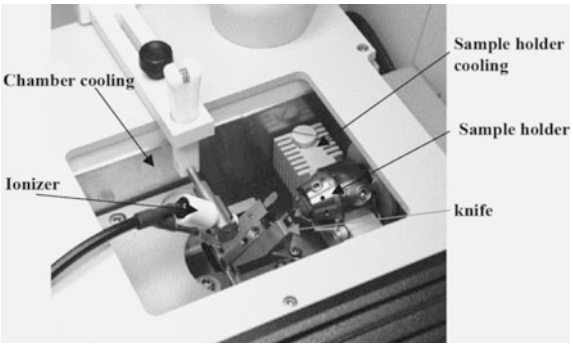

Fig. 4.96 Special chamber for cryo-ultramicrotomy

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.

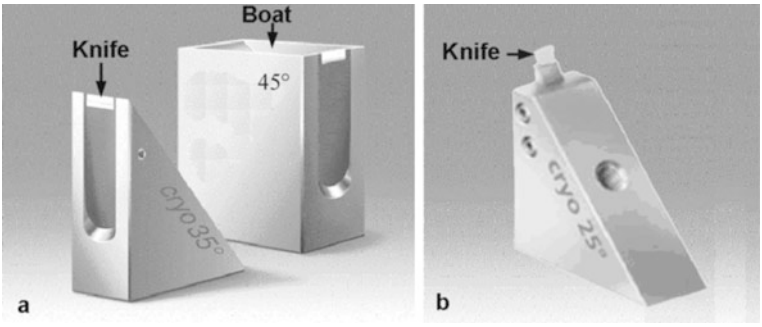

Fig. 4.97 **a** and **b** Diamond knives for cryo-ultramicrotomy: (**a**) 45 and 35° and (**b**) 25° angle, allowing least possible compression

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

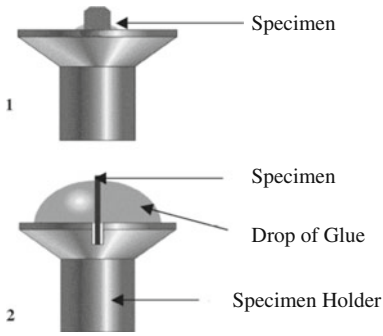

Fig. 4.98 Cold specimen holders

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

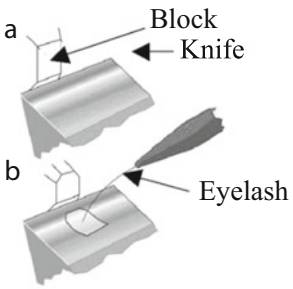

Fig. 4.99 Making a dry cut

Fig. 4.100 Recovering dry sections

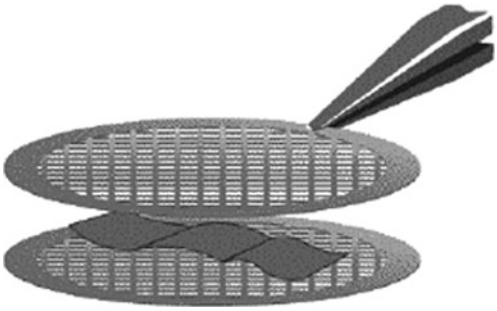

Fig. 4.101 Pressing sections to make them adhere to the grid

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.

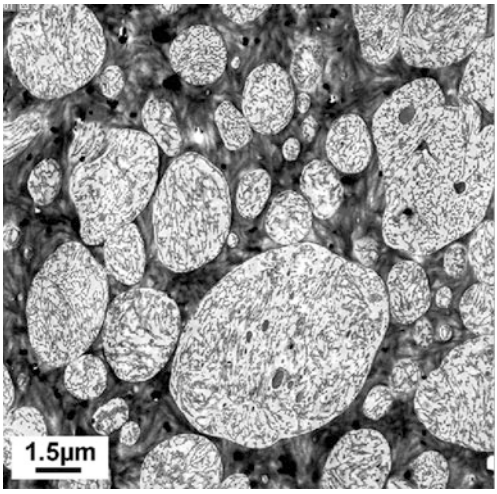

Fig. 4.102 Mixture of polyamide and PPE polymers (*H. Gnaegi, Diatome, Bienne*)

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.

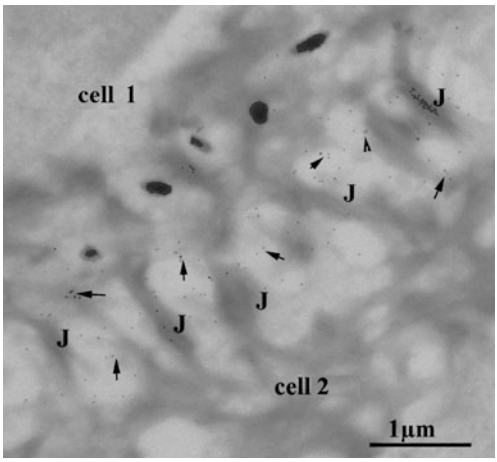

Fig. 4.103 Junction between two epidermal cells called keratinocytes. Immuno-localization of an epidermal proteoglycan located at the spike junctions between two cells. The gold labeling particles are 10 nm in size. J: intracellular junction; *arrows* point to areas of labeling (*M. Hafetek, EA3732,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

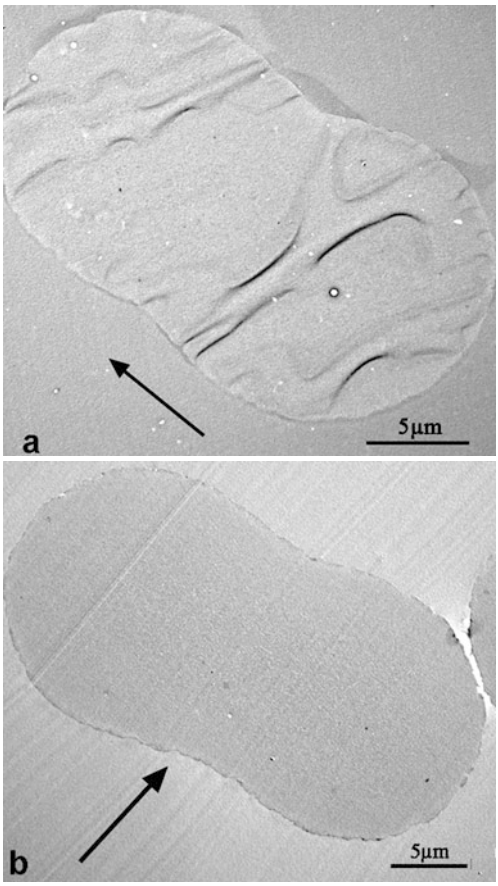

Fig. 4.104 (**a**) Copolymer cut at room temperature. The *arrow* indicates the cutting direction. (**b**) Same copolymer cut using cryo-ultramicrotomy. The *arrow* indicates the cutting direction. The polymer nodule, which is softer than the matrix, folds during cutting at room temperature, while it 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.

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Chapter 5
Replica Techniques

1 Direct Replica Technique

1.1 Principle

The direct replica technique is used to make an impression of the surface topography of a sample that cannot be observed directly in the microscope because it is too large or too thick. The resulting replica gives an inverted topography image.

A thin carbon film deposit is used for the mold.

This technique is performed using a combination of physical and chemical reactions: the first step includes a series of physical actions involving shadowing by metal deposition under low incidence, followed by carbon film deposition; the second step uses a chemical reaction to dissolve the sample.

This technique is performed on large bulk materials and on materials sensitive to the electron beam, such as some polymers. It is indispensable for investigating radioactive materials, but is not applied to chemically inert materials.

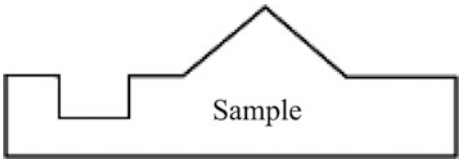
1.2 Operating Mode

1.2.1 Equipment and Supplies

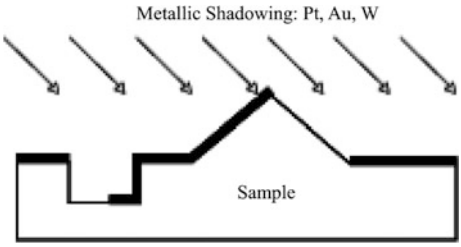
The necessary equipment includes an evaporator under high vacuum containing a specimen holder with various orientations and two evaporation sources, one for metal and the other for carbon.

J. Ayache et al., *Sample Preparation Handbook for Transmission Electron Microscopy*,
DOI 10.1007/978-1-4419-5975-1_5, © Springer Science+Business Media, LLC 2010

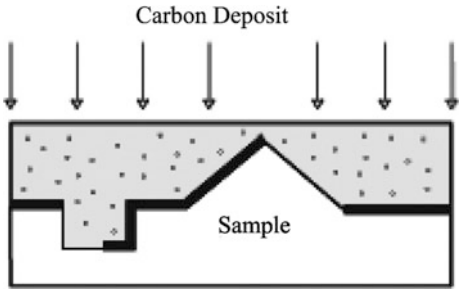
1.2.2 Procedure



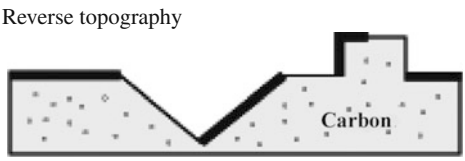
Step 1: The sample's surface is first cleaned, and then the sample is placed in an evaporator equipped with two evaporation sources: one for metal and the other for carbon.



Step 2: Shadowing of the surface sample is performed to reproduce the topography of the surface roughness. Once the vacuum conditions are reached i.e., 10^{-3}–10^{-4} Pa, the metal (Pt, Au, W, etc.) is deposited under an incidence angle ranging between 5° and 30°.



Step 3: While maintaining the vacuum in the evaporator, an amorphous carbon film is deposited perpendicular to the sample surface. This film forms the molding and acts to reinforce the shadowing film.



Step 4: The sample is removed from the evaporator and is dissolved using chemical etching with a corrosive solution appropriate for the sample type. When the sample is completely dissolved (dissolution time varies widely depending on the thickness and initial sample type), the replica is recovered after several rinses with distilled water.

Transferring the Replica onto the Support Grid

When the sample is completely dissolved, the replica made from the metal film and consolidated by the carbon film is recovered using a corner of filter paper or a special tool called a "perfect loop," like the tool used to pick up ultramicrotomic thin sections. To perform an initial rinse, the replica is then transferred to the surface of a beaker containing distilled water. To limit the damage caused by the surface tension of the distilled water, a few drops of alcohol or acetone can be added. The operation is repeated once or twice depending on the nature of the sample and, above all, depending on the concentration of the chemical solution used for dissolving the sample.

In the final rinse, the replica fragments are recovered by capillary action or by fishing them out from below the water surface using a support grid. To prevent recovered fragments from being superimposed onto one another, the underside of the support grid is wiped delicately with paper towels. The grids are then dried before observation under the microscope (Fig. 5.1).

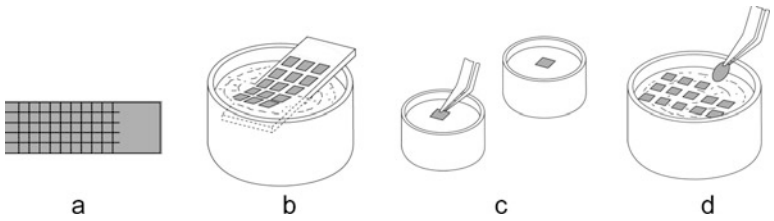


Fig. 5.1 Replica transfer on the support grid: (**a**) squared scarification on the carbon film; (**b**) substrate dissolution by chemical etching leading to fragments detaching from the surface; (**c**) successive rinses with distilled water; (**d**) replica recovery on the TEM support grid

When the topography of the sample allows, the sample can be cut into small 3-mm square pieces using a tip or a scalpel. These squares will detach and float on the surface of the chemical etching solution. They are then recovered one by one, rinsed several times, and then each one is deposited on a support grid.

Since the surface to be studied is large, it is generally prudent to recover several grids from the same sample, enabling many different observations to be performed.

Figures 5.2 and 5.3 show the different applications of the technique for the two types of materials.

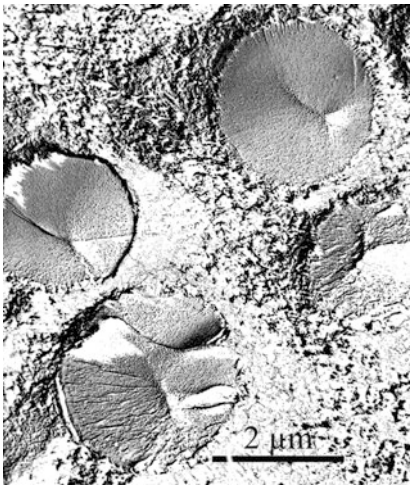


Fig. 5.2 Barium borate glass, globules of a phase rich in BaO in a matrix rich in B_2O_3. Direct replica with palladium shadowing (*G. Ehret, EOST Strasbourg*)

Fig. 5.3 Replica of an aluminum crystal embedded in a vitreous phase. Direct replica with platinum shadowing (*P. Peter, EOST Strasbourg*)

1.3 Variants

This technique has several variants.

1.3.1 Carbon Film Variant

A simplified variant of the preparation process is based on the use of a single carbon source for both steps: shadowing and molding. Carbon shadowing is often of a very high quality because the film presents neither texture nor significant granular morphology. The resulting shadowing effect is then somewhat diminished.

1.3.2 Metallic Film Variant

In this case, a single mixed evaporation source composed of a hollow graphite electrode with a metal core (Pt+C, Ir+C, etc.) is used. The metal and carbon are evaporated simultaneously and deposited under a low incidence angle. This variant is applied to samples with low surface roughness.

Figures 5.4 and 5.5 illustrate an application of this technique in the field of synthetic minerals presenting topography with low surface roughness.

1.4 Advantages

This technique is indispensable for investigating materials that are very sensitive to the electron beam or other radioactive materials. For radioactive materials, preparation can be carried out in protective enclosures. Because the final mold is not radioactive, TEM observation does not require any special precautions.

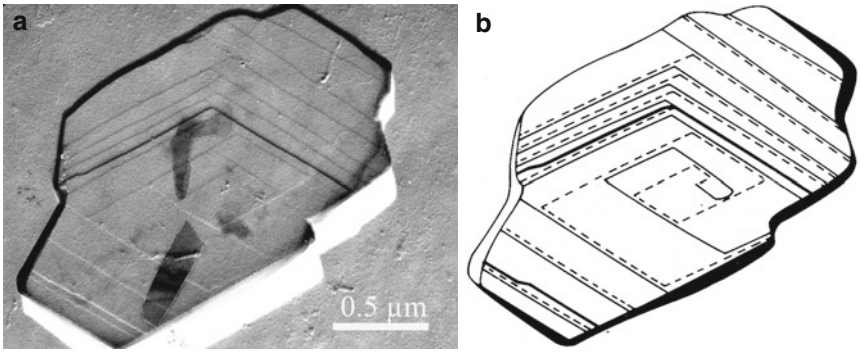


Fig. 5.4 Interlocking spiral in two layers observed on synthetic muscovite, producing a $2M_1$ structure. The growth step is 1 nm high, which represents a mica flake. This sample is a direct platinum–carbon replica prepared using the variant adapted to low surface roughness topography. (**a**) TEM image. (**b**) Representation diagram illustrating the double spiral (*M. Amouric, CRMC-N Marseille*)

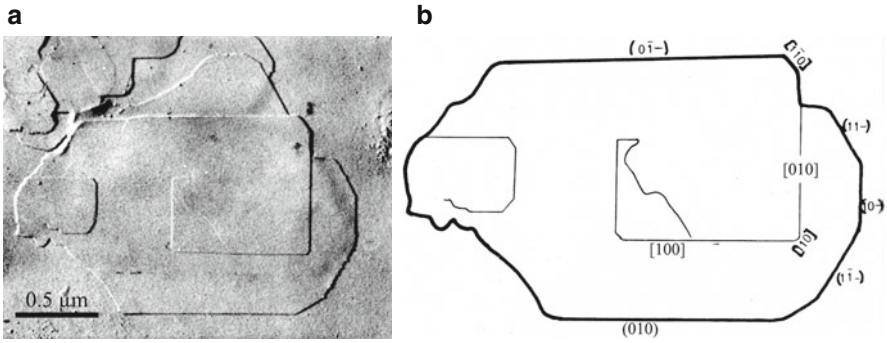


Fig. 5.5 Platinum–carbon replica of synthetic muscovite with very low surface topography. **(a)** TEM image showing a multiple spiral on a base structure. **(b)** Representational diagram of the different crystal planes (*M. Amouric, CRMC-N Marseille*)

1.5 Limitations

The major limitation is the destruction of the sample, which is dissolved in the final step of the preparation process. Consequently, this technique is not used to investigate precious or rare samples, such as valuable minerals, collection specimens.

This technique requires knowing which chemicals are capable of dissolving samples without damaging replicas. The right combination is often difficult to find. This technique is limited to observing topography and produces a reverse image of the sample surface, making interpretation difficult. However, this technique is difficult to use for samples presenting a high surface topography roughness.

1.6 Artifacts

This technique does not present artifacts. Poor mold quality or tearing of the film is due to poor knowledge of the technique procedure; these types of artifacts are easily recognizable.

1.7 Type of Analysis

Topography.

1.8 Risks

The only risks encountered by the operator are chemical in nature: corrosive solutions used to dissolve the sample can cause chemical burns. It is essential to work under an extractor hood.

1.9 Conclusion

Although in the past this technique was quite widespread in laboratories, nowadays its use has fallen drastically. Currently, high-resolution scanning electron microscopes and environmental scanning electron microscopes allow the direct observation of a sample′s topography. This technique remains highly useful for investigating dangerous materials such as radioactive materials.

2 Indirect Replica

2.1 Principle

The indirect replica technique is used to make a topographical print of the surface via a twin replication of a sample that cannot be observed directly in the microscope because it is too large or too thick.

Molding requires an initial deposition of a thin carbon film or polymer film, followed by counter-molding with carbon: Counter-molding is the equivalent of a direct replica.

This method uses a succession of physical actions such as molding and counter-molding, followed by a mechanical action when the molding is removed, and finally a chemical action to dissolve the molding.

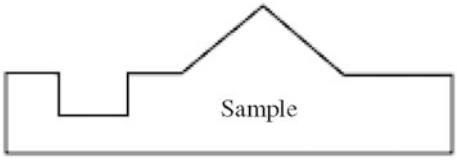
This technique is performed on compact or porous, single-phase or multiphase bulk materials. It is often used for large samples or samples sensitive to the electron beam and is an indispensable technique for materials that must be kept intact.

2.2 Operating Mode

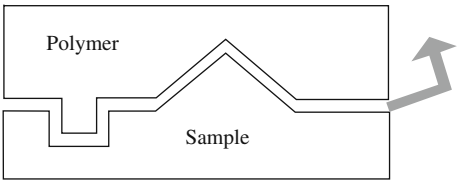
2.2.1 Equipment and Supplies

The equipment consists of an evaporator under secondary vacuum containing a tiltable specimen holder and two evaporation sources, one for the metal and the other for carbon.

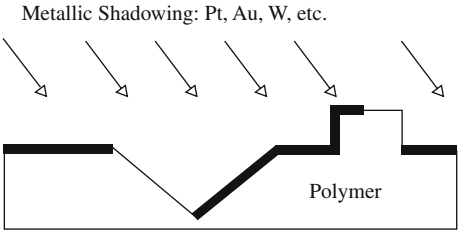
2.2.2 Procedure



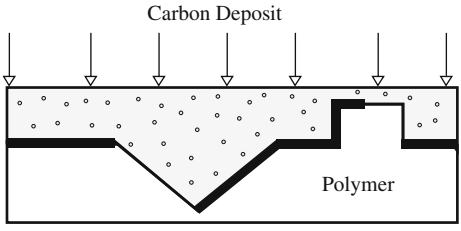
Step 1: Before the first molding, the initial sample surface must be cleaned.



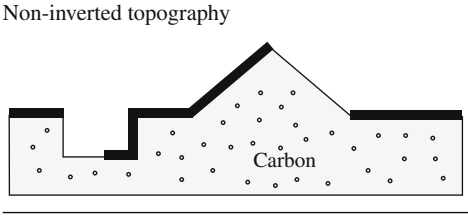
Step 2: The first mold of the sample surface is taken using a liquid polymer or a polymer film soaked with a solvent that is applied on the sample surface to be replicated. This molding is then pulled away from the surface.



Step 3: To produce shadowing on the mold of the first film removed, vacuum conditions must reach 10^{-3}–10^{-4} Pa. Then, a 10-nm-thick layer of metal (Pt, Au, W, etc.) is deposited under an incidence angle ranging between 5° and 30°.



Step 4: While maintaining the vacuum in the evaporator, an amorphous carbon film is deposited perpendicular to the sample surface. This film forms the counter-molding and reinforces the shadowing film.



Step 5: The sample is removed from the evaporator and placed onto microscope support grids, where the polymer mold is dissolved.

The resulting replica provides a non-inverted topography of the initial sample.

Dissolving the Polymer Molding

In general, the sample, which is composed of the polymer mold reinforced with a carbon film, is deposited onto metallic mesh support grids. Then the entire set is placed in a container to which solvent is added, generally acetone, until flushing occurs. Dissolution occurs by capillary action, as shown in Fig. 5.6.

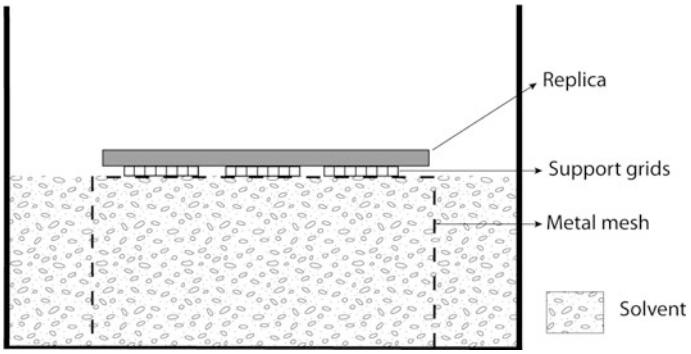


Fig. 5.6 Diagram of the setup used for the polymer mold dissolution

The polymer film dissolves very quickly and does not require any special precaution.

A scalpel can also be used to cut 3-mm square pieces, as well as to recover polymer molds on the grids, one at a time, before proceeding with dissolution (as previously described).

It is important to avoid placing the replica support grids on filter paper before they are completely dried, as it may break or crumple the replica.

The replica can then be observed. If any trace of polymer remains, the grid can be placed in contact with the solvent until complete dissolution and then dried.

2.3 Variants

There are no variants.

2.4 Advantages

This technique is non-destructive. It is indispensable for investigating samples that must be kept intact, such as precious objects, archeological specimens, or items with great artistic value from museums. It can be applied to chemically inert materials, large samples, and specimens that cannot be moved. The use of indirect replicas makes it easier to interpret the surface topography image of a sample, compared to the direct replica technique.

2.5 Limitations

Making the first molding with a polymer and tearing it from the sample surface can disrupt the topography and reduce the final resolution of the resulting image.

2.6 Artifacts

There is a risk of change or damage to the initial morphology, which can lead to a misinterpretation.

2.7 Type of Analysis

Topography.

2.8 Risks

The only risks encountered by the operator are chemical in nature: Corrosive solutions used to dissolve the polymer film can be noxious. It is essential to work under a fume hood.

2.9 Conclusion

Although in the past this technique was quite widespread in laboratories, nowadays its use has fallen drastically. Currently, high-resolution scanning electron microscopes and environmental scanning electron microscopes allow the direct observation of a sample′s topography. However, the indirect replica technique remains very useful when investigating rare or precious materials that cannot be destroyed or items that must be handled with great care (such as materials displayed in an exhibition). The same goes for very large objects that cannot be brought into laboratories.

3 Extractive Replica

3.1 Principle

The principle of this technique is based on the *extraction* of particles from the surface of a sample too thick to be observed directly in TEM. The particles are trapped in a carbon or polymer film in order to be separated from the substrate, which is then chemically dissolved.

This technique is performed on compact or microporous, single-phase or multiphase bulk materials. It is mainly used to investigate precipitates in a metallic matrix,

catalytic particles deposited on ceramics or glasses, and nanomaterials produced by crystal growth on bulk substrates.

3.2 Operating Mode

3.2.1 Equipment and Supplies

Performing this technique involves the use of either an evaporator under high vacuum for making the carbon film or a polymer in solution or a polymer film.

3.2.2 Procedure

Procedure 1

For bulk materials, the particles to be extracted are found either on the sample surface or inside the matrix. They must be uncovered using chemical, electrochemical, or ionic etching.

This technique is mainly used to investigate bulk materials such as metals and steels. In this case, a preliminary polishing of the sample surface is first performed (Fig. 5.7a). Polishing can be mechanical, electrolytic, or chemical. The particles trapped in the matrix must be highlighted by an appropriate chemical etching (Fig. 5.7b).

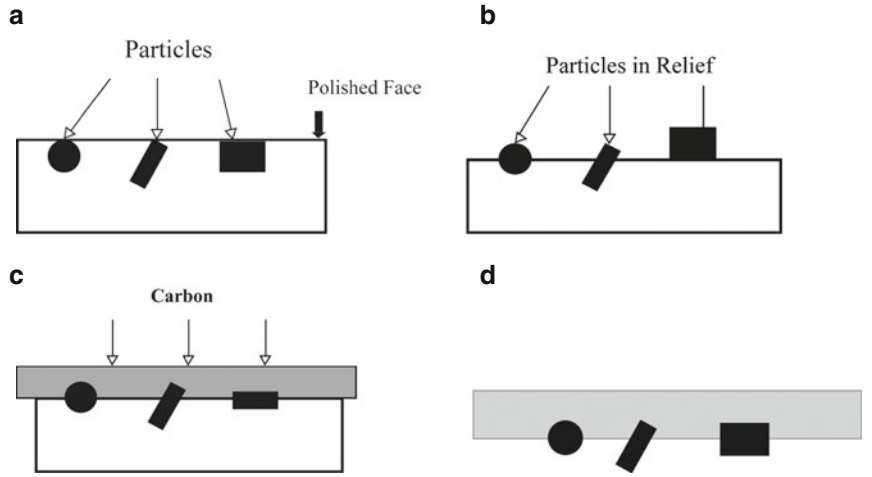


Fig. 5.7 (**a**) Initial sample; (**b**) sample after chemical etching; (**c**) carbon deposit; and (**d**) final replica

Then a carbon layer between 10 and 30 nm thick is deposited by evaporation under high vacuum. A selective chemical etching of the substrate releases the carbon film containing the particles to be observed (Fig. 5.7c). They are in relief and trapped in the amorphous carbon film (Fig. 5.7d).

Before dissolution of the substrate and with the use of a hard tip, the carbon film is cut into 3-mm square pieces by scarification. The selective chemical etching of the substrate causes the small replica squares to detach and float on the solution surface, as shown in Fig. 5.8. Because of the corrosive nature of the chemicals, the use of a fume hood and protective goggles is absolutely mandatory.

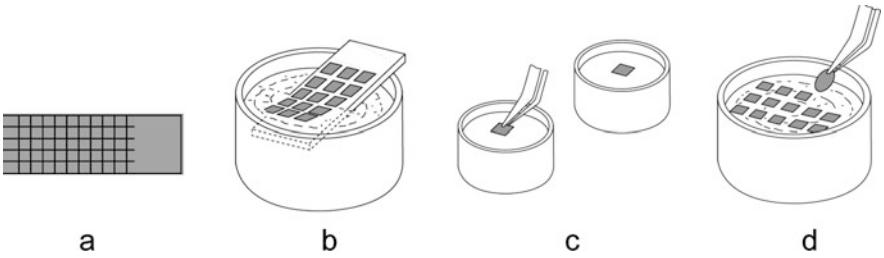


Fig. 5.8 Replica transfer on the support grid: (**a**) scarification of the squares on the carbon film; (**b**) substrate dissolution by chemical etching, leading to fragments detaching from the surface; (**c**) successive rinses with distilled water; and (**d**) replica recovery on the TEM support grid

Rinses are performed with distilled water contained in Teflon vessels; a minimum of 5% acetone is added to lower the surface tension of the water and prevent the carbon film from tearing when coming into contact with the water (Fig. 5.8b). Tabs of filter paper, cut into triangles, are used to transfer the small squares from the corrosive solution to the rinse water. There are special tools available in the form of a ring at the end of a rod (perfect loop) that can be used to handle very fragile objects. Rinsing time is between 2 and 3 min and a minimum of three rinses is necessary (Fig. 5.8c). After the last rinse, the obtained replica can be recovered onto the support grids (Fig. 5.8d). After the successive washings, the replicas are dried before observation on an adequate support (never directly on filter paper as the replica would stick to the paper via capillary action).

This technique is also useful to investigate nanoparticles produced by the chemical vapor deposition (CVD) of thin layers on a bulk substrate. For example, diamond-type layers or carbon nanoaggregates on copper or silicon single-crystal substrates (Fig. 5.9) can be studied using this technique.

To investigate diamond layers or carbon nanoaggregates deposited on a copper single crystal, the chemical solution used for etching contains 100 g of trichloroacetic acid dissolved in 500 ml of ammonia and 500 ml of distilled water. After depositing the thin carbon film by evaporation under vacuum and cutting small 3-mm square pieces by scarification, successive washings in distilled water are carried out as in the previous procedure. The washed squares are then recovered onto support grids in order to be observed (Fig. 5.8).

Procedure 2

For fine particles, the grains containing the matrix and the micro- to nanometer-sized metallic particles are first dispersed on the surface of freshly cleaved mica slide

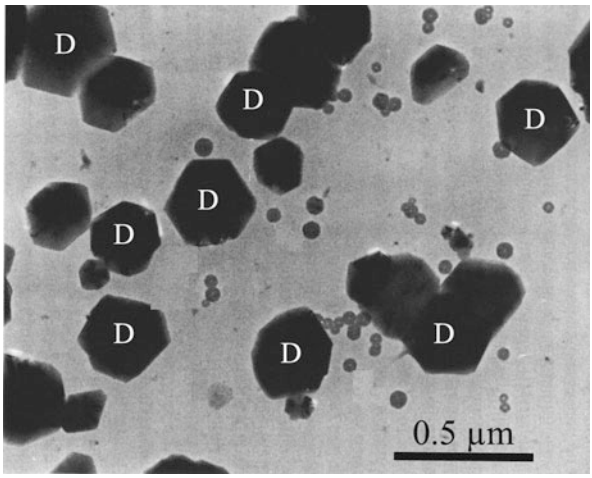


Fig. 5.9 Diamond layer obtained by CVD on a copper single crystal. Hexagonal diamond crystals are marked with a "D." The smaller, round particles are "onion-like" carbon phases (*F. Le Normand, IPCMS, Strasbourg*)

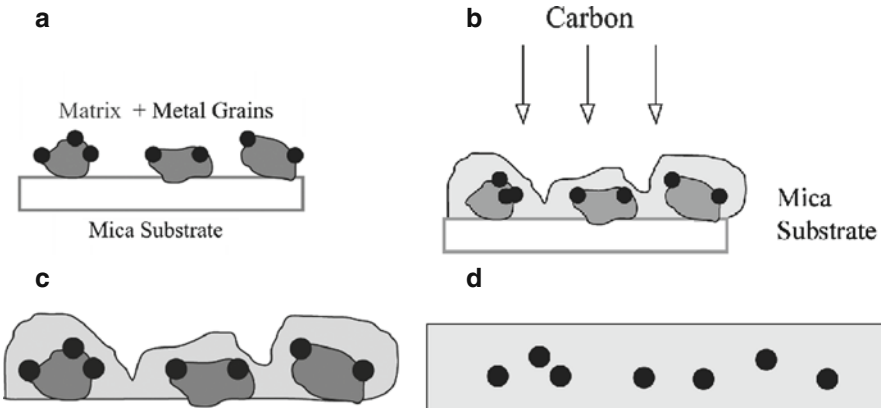


Fig. 5.10 (**a**) Initial sample deposited on the surface of the freshly cleaved mica slide; (**b**) carbon deposit; (**c**) dissolution of the substrate and matrix; and (**d**) final replica

(Fig. 5.10a) A carbon film is then deposited (Fig. 5.10b) on the dispersed grains to coat the matrix and the metallic particles. Then the carbon film is cut into squares by scarification, as in the previous procedure.

The square-replica fragments must then be separated from the mica substrate, and the matrix should be dissolved by plunging the substrate-replica into a solution containing a mixture of water, acetone, and hydrofluoric acid. The acid concentration

must be chosen so that it etches the matrix without dissolving the metallic particles (Fig. 5.10c). The selective chemical etching of the substrate causes the small replica squares to detach and float on the solution surface, as can be seen in Fig. 5.8b. In this case, the matrix must be completely dissolved in order to retain only the particles to be investigated (Fig. 5.10d). This requires long etching times of several hours (4–24 h), with regular sampling in order to check the process.

If the matrix is not sufficiently dissolved, it is difficult to distinguish the metallic particles from the grain of the matrix. In this case, resolution is poor because the metallic particles superimposed on the remaining matrix are too thick. On the other hand, when the matrix is completely dissolved, its contour is still seen through the shadowing effect (Fig. 5.11), and the metallic particles appear clearly because they have higher contrast due to their higher atomic number (*Z*) (Fig. 5.12). This technique is widely used to investigate catalysts, e.g., metallic grains (Pt, Pd, etc.) supported on an alumina-based (Al_2O_3) or ceria-based (CeO_2) matrix (Figs. 5.11 and 5.12).

In order to dissolve the alumina grains, 500 ml of the following chemical solution is prepared: Start with 325 ml of distilled water and then add 125 ml of fluorhydric acid titrated at 40% and 50 ml of acetone. *Caution:* The order in which each item is added to the solution is critical. Water first, then HF, then acetone at the end. *The mixture of acetone + HF is explosive!* Fluorhydric acid is very corrosive to both metals and glass; therefore, Teflon vessels and tweezers coated with Teflon or another inert metal such as platinum must be used.

Because of the corrosive nature of the chemical used, the use of a fume hood and protective glasses is absolutely mandatory.

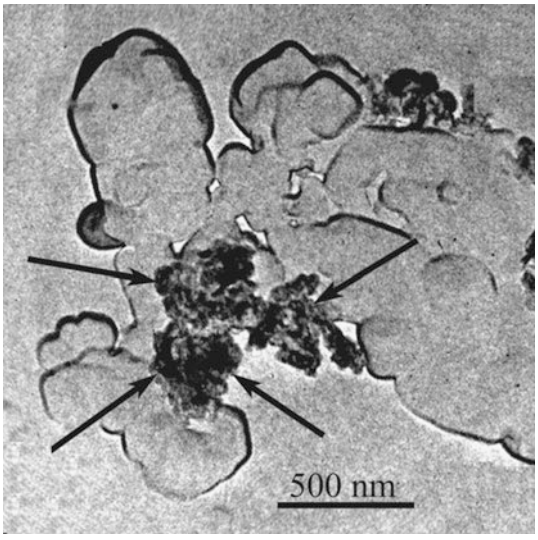


Fig. 5.11 Extraction replica of a fine particle material: Pt catalyst on alumina. The alumina grain is completely dissolved, only the carbon film with the metallic particles in the center, indicated by the *arrows*, is left behind (*G. Ehret, IPCMS, Strasbourg*)

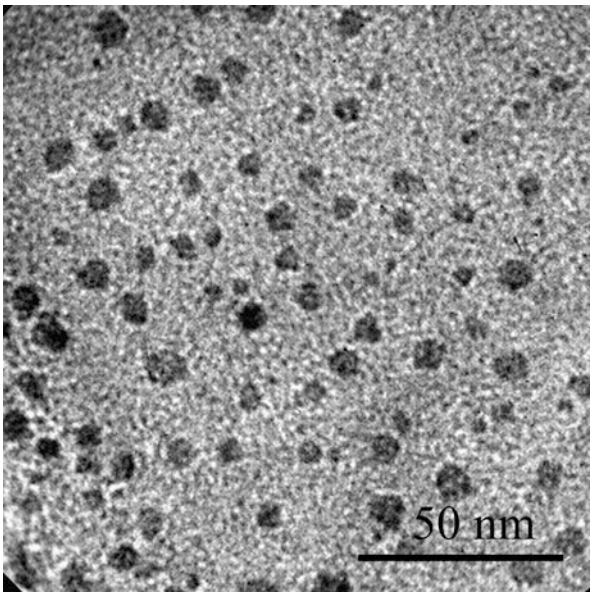


Fig. 5.12 High-magnification image of the same area of the replica. We can very clearly see the metallic particles of the catalyst are well dispersed and homogeneous in size (*G. Ehret, IPCMS, Strasbourg*)

Dissolution is performed in Teflon containers, as in procedure 1 (Fig. 5.8). The squared pieces are rinsed in distilled water containing 5% acetone. Replica samples will be picked up successively at different time intervals ranging from 4 to 24 h and will be observed under TEM in order to determine the best dissolution time to analyze only the particles extracted.

3.3 Variants

3.3.1 Two-Step Carbon/Plastic Extraction Replica Technique

This non-destructive technique is used to keep the sample surface intact, with the exception of extracted particles or in the case of substrates that are difficult to dissolve.

As in the general example in procedure 1, the initial sample presents sides that are polished using mechanical, electrolytic, or chemical polishing (Fig. 5.13a). The particles trapped in the matrix must be highlighted by an appropriate chemical etching (Fig. 5.13b).

Then the mold is made either by depositing a plastic material that can be in the form of a plastic sheet a few millimeters thick that has been pre-softened in an adequate solvent or by pouring a liquid plastic directly on the surface (Fig. 5.13c). After

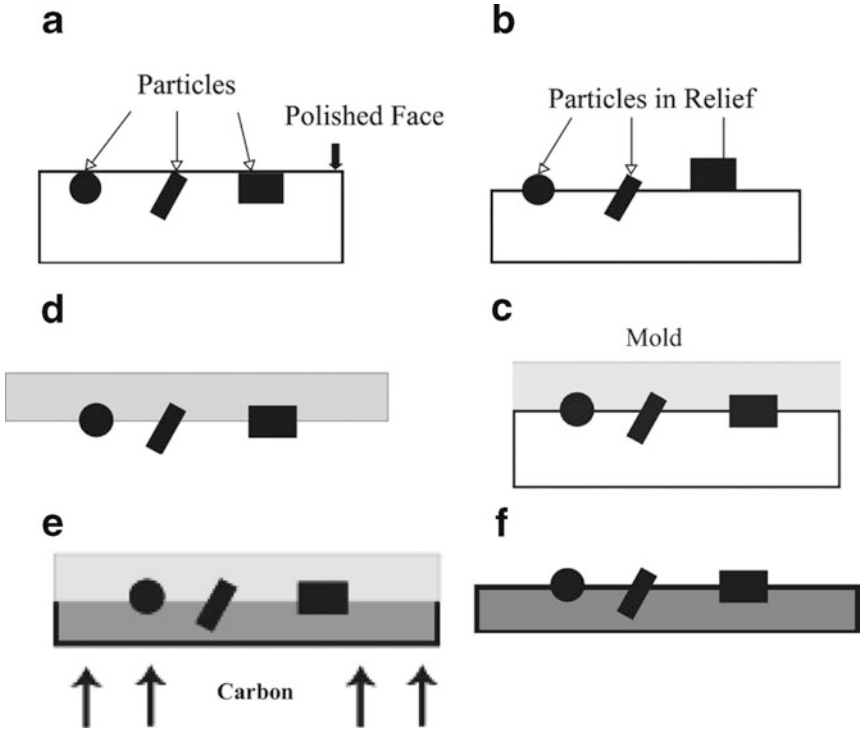


Fig. 5.13 (**a**) Initial sample; (**b**) sample after chemical etching; (**c**) plastic impression; (**d**) unsticking the sample; (**e**) carbon deposit; and (**f**) final replica obtained after dissolution of the plastic film

drying, this imprint is mechanically removed from the surface and now contains particles that are unstuck and retained in the plastic film (Fig. 5.13d).

Next, amorphous carbon is evaporated under high vacuum (Fig. 5.13e) and the plastic film is dissolved after being cut into small squares that are the same size as the support grid (Fig. 5.13f). Since the plastic film is thin, it dissolves very quickly.

The plastic film is dissolved directly on the support grid, which is placed in contact with the solvent but not immersed in it (Fig. 5.14). Usually, the grids are placed on a porous support or on a grid with large mesh in a Petri dish and are flushed with solvent. Dissolution occurs by capillary action. The grid must not be immersed in the solvent because the carbon film might break.

There are many substances available for making plastic impressions: biodene, rhodoid, Triafol, Jacquet varnish, etc.

Generally, acetone is used as the solvent: For biodene, a mixture of 100 ml of dichloromethane and 20 ml of methanol is used for 2 h, followed by a second rinse in acetone, also for 2 h.

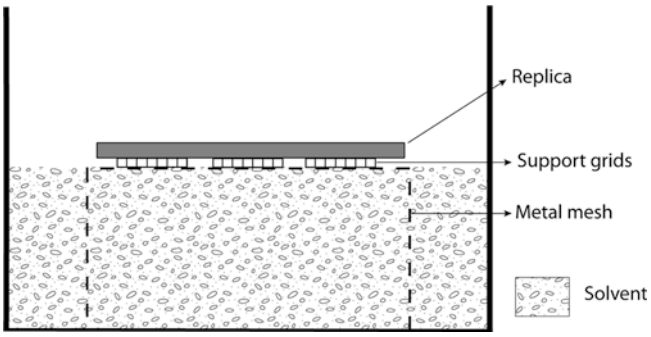


Fig. 5.14 Diagram of the setup to use for plastic film dissolution

3.4 Advantages

This technique can be used to obtain a large number of grids simultaneously prepared for the same sample. It facilitates the use of statistical analyses on particle size, shape, structure, and chemical composition.

3.5 Limitations

The major drawback is the poor control of chemical dissolution. This problem is overcome by choosing different chemical etching times and comparing the results. Eliminating the matrix prevents investigating the particle–matrix interaction. This technique is difficult to implement when the matrix is very rough.

3.6 Artifacts

Artifacts are directly related to the problem of poorly controlled chemical dissolution, as these result in a change in particle size.

3.7 Type of Analysis

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

3.8 Risks

The only risks encountered are chemical in nature: Corrosive solutions used to dissolve the sample can cause chemical burns or intoxication by noxious vapors. It is essential to work under a fume hood.

3.9 Conclusion

This technique is critical for the statistical or chemical analysis of particles isolated from their matrix. It is very useful for investigating precipitates in a matrix, as well as the latest-generation nanomaterials obtained by CVD (chemical vapor deposition).

4 Freeze Fracture

4.1 Principle

The principle behind this technique is to make a topographical print resulting from a fracture in a frozen hydrated sample. The replica is equivalent to a thin slice.

Samples should be very small (approximately 1 mm^3) so as to get ultrarapid freezing at the sample core. Fracturing is performed at 123 K and can be single or double. A single fracture is started using a metallic knife on the sample surface. A double fracture is obtained by abruptly opening a sandwich. This is done by clamping the sample between two copper or gold metal cupels. Fracturing is followed by metallic shadowing with platinum, at an angle of 45° with regard to the sample surface. Finally, the sample is coated with a carbon layer (90° angle). Shadowing and carbon coating occur at 123 K under an ionic vacuum of 10^{-5} Pa.

Once the sample replica is back to room temperature, the sample is dissolved using an appropriate chemical solvent. The platinum–carbon replica is then rinsed several times, before being mounted on a TEM support grid.

This technique can be performed on bulk hydrated materials as well as fine particles in suspension.

4.2 Operating Mode

4.2.1 Equipment and Supplies

This involves a chamber with a high vacuum, between 10^{-5} and 10^{-6} Pa, that can be temperature regulated up to 103 K. The vacuum is produced using cascading pumps, the last one being an ion pump (Figs. 5.15, 5.17, 5.18, and 5.19). Vacuum quality is essential to correctly perform this technique. A perfectly clean (no oil residue) and dry vacuum is required. The best results are obtained with equipment containing absorption pumps that do not generate oil vapors or turbo-molecular pumps with magnetic bearings. The high vacuum is provided by an ion pump.

The chamber is equipped with an airlock system (8 in Fig. 5.15) for inserting and removing the sample. The chamber also contains metal and carbon (14 and 15, respectively, in Fig. 5.15) evaporation sources, since the best results are obtained using electron guns.

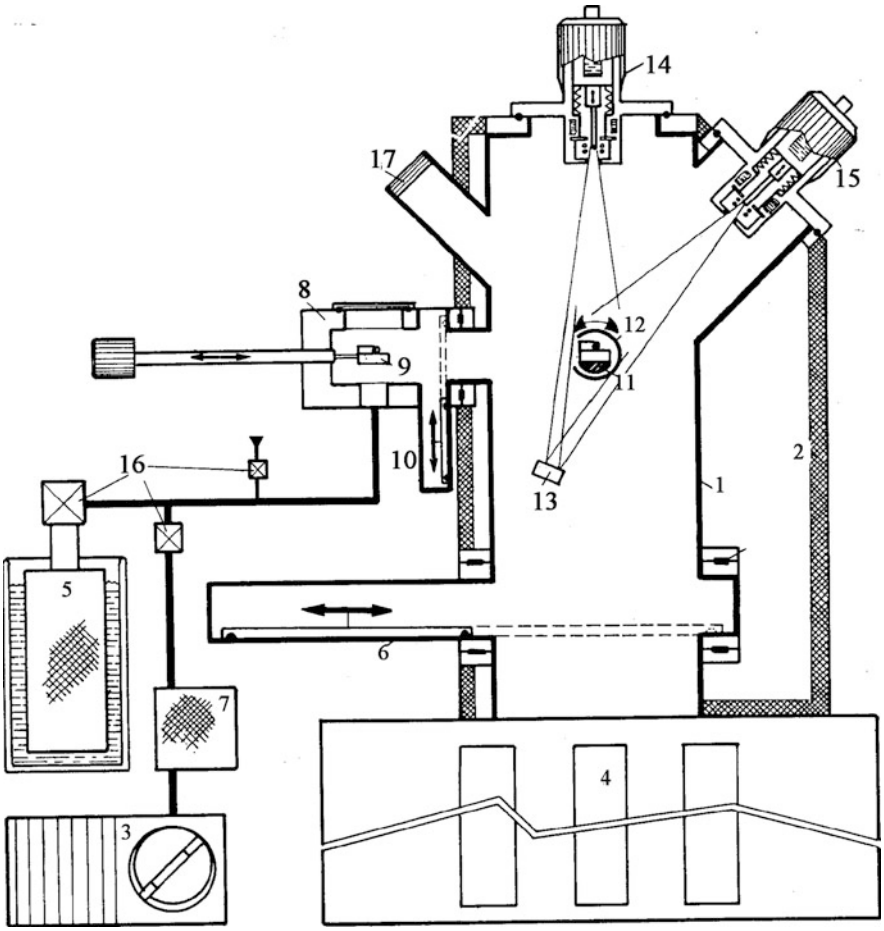


Fig. 5.15 Diagram of a freeze-fracture device: 1,chamber; 2, heater unit; 3, rotary pump; 4, ionic pump; 5, sorption pump; 6, gate valve; 7, molecular sieve filter; 8, introduction airlock; 9, fracturing specimen holder; 10, airlock gate valve; 11, specimen holder stage; 12, trap cylinder; 13, oscillating quartz; 14, platinum source; 15, carbon source; 16, electromagnetic valves; 17, porthole

The gun used for shadowing contains a small piece of platinum inserted into a hollow carbon electrode. The tungsten filament surrounding the electrode is charged with an electrical current, causing the extraction of the platinum atoms. These atoms are directed toward the sample because of a difference in potential and a system of electrically charged plates located around it. This device improves the collection and orientation of particles evaporated toward the sample.

The gun used for the carbon replica contains a single solid carbon electrode.

The presence of an oscillating quartz crystal (13 in Fig. 5.15) in the chamber enables precisely measuring the thickness of the metal or carbon coating.

In the center of the chamber is a stage (11 in Fig. 5.15) that receives the specimen introduced through the airlock (8 in Fig. 5.15) using a long rod. The stage is inserted in a cylinder (12 in Fig. 5.15) that opens the specimen holder as it turns. This cylinder, which can be oriented with regard to the two electron beam guns, is also a trap for sublimation.

4.2.2 Procedure

Two techniques can be used depending on the type of device.

Sandwich Technique with Double Cupels

The sample is placed into a metal cupel with a 3-mm internal diameter. There are depth carriers for bulk samples, semi-depth carriers for thick or viscous suspensions, and flat carriers for fluid suspensions. These cupels are adapted to the device in which the cryo-fracture technique will be performed and are placed in pairs for making a sandwich (Fig. 5.16). Regardless, the sample must be small enough to ensure proper freezing.

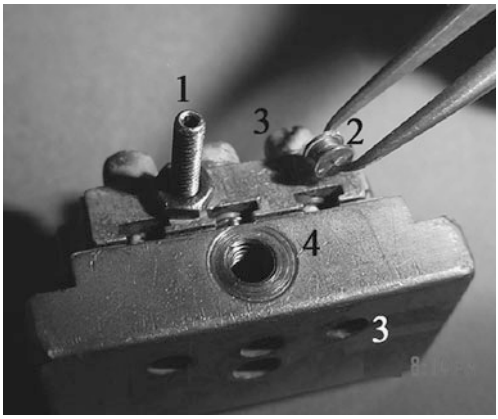


Fig. 5.16 Closed support for placing three sandwiches (2) clamped in a dovetail box with opposite screws (3). The threaded rod (1) is turned down in the device by the adjustable cylinder (11 in Fig. 5.15) to open the box; the orifice (4) is used to thread a tool that provides the introduction of the support into the device

Sandwiches are frozen before being introduced into the device's chamber, either in liquid nitrogen, slush nitrogen, or in propane or ethane cooled with liquid nitrogen or, better still, in propane cooled to 98 K. With special equipment, freezing can be performed under high pressure for better results (see "Methodology", Chapter 4 and "Techniques", Chapter 2, Section 12).

Sandwiches are transferred into the device support under liquid nitrogen. This is a delicate step in the technique that requires some experience, as the cupels and the support should not come into contact with the ambient air. Ambient air always

contains humidity that gets trapped on very cold surfaces, resulting in frost. The presence of frost prevents the proper placement and therefore the subsequent opening of the cupels in the support. The support must also be locked in the closed position, so as not to cause the sample to fracture before it is inserted in the device. Using the tool in position 4, the support is introduced into the device airlock as quickly as possible, in order to prevent frost from forming on its walls. After primary and secondary pumping, it is introduced into the chamber.

After a period of vacuum stabilization at around 10^{-5} Pa and temperature stabilization at around 123 K, rod 1 is switched by turning the cylinder containing the support and the cupels open, causing the sample to fracture (Fig. 5.17).

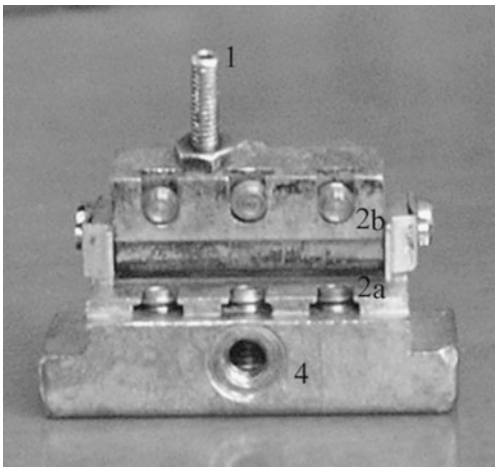


Fig. 5.17 The same support opened, there is a cupel on each side (2a and 2b) containing the specimen whose fractured surface will be shadowed and then carbon coated

The specimen holder is placed so that it will receive platinum shadowing at a 45° angle with an electron gun. The angle can be adjusted depending on the relief to be highlighted. The layer of platinum is on the order of 10 nm thick. Using a second gun, under a 90° angle, specimen is coated with a 100–200 nm carbon layer. The sample is then removed from the device.

The succession of steps is summarized in the diagram provided in Fig. 5.18.

Cleaning the Replica

The preparation may be consolidated by the deposition of a fine collodion film on its surface (the collodion is in solution at 1% in isoamyl acetate). At room temperature, and under a fume hood, the sample is then unglued from the support and floated on the surface of a liquid containing a dissolution agent (acid, bleach, detergent, or solvent) (Fig. 5.19a). A dissolution time must be chosen that is compatible with the mechanical resistance of the replica's carbon film.

Dissolution lasts between 10 min and several hours depending on the type of material.

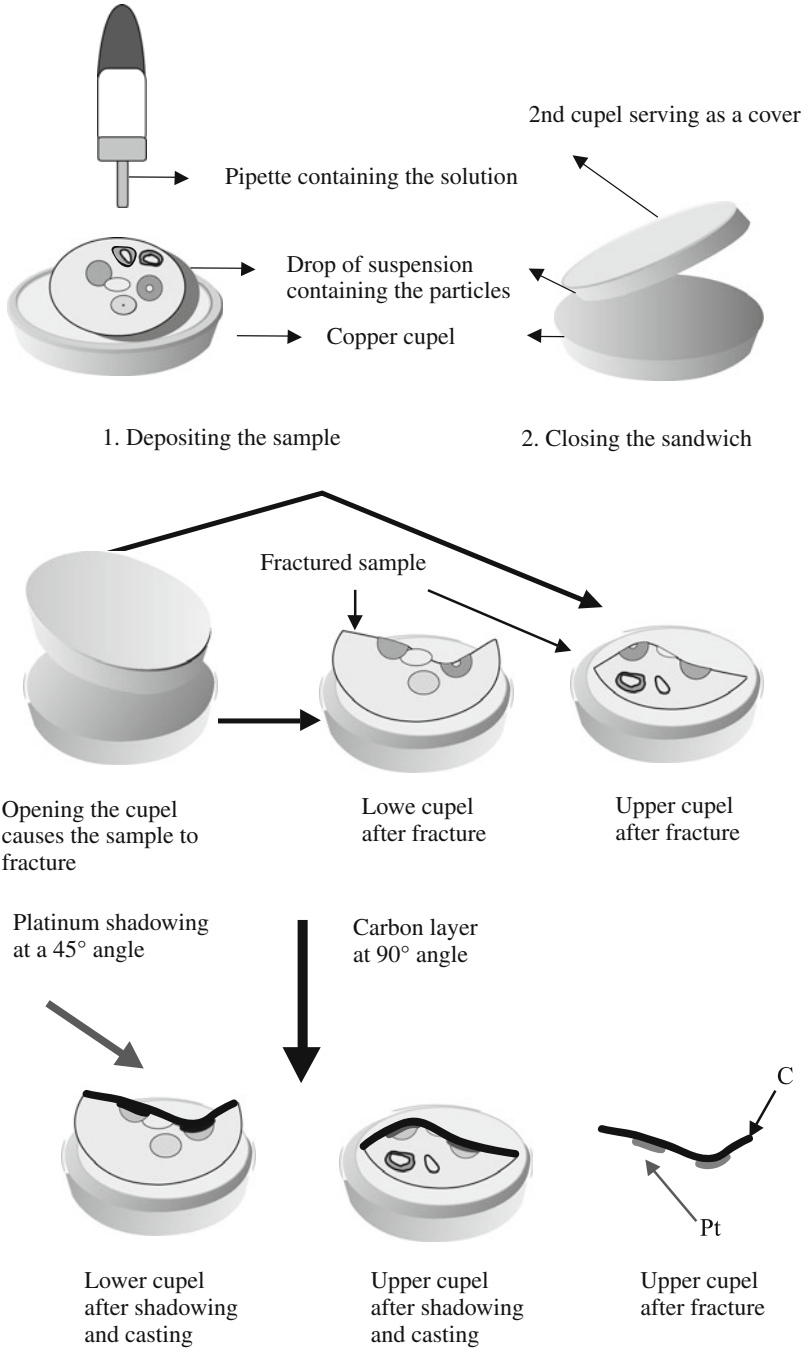


Fig. 5.18 Diagram of the succession of steps

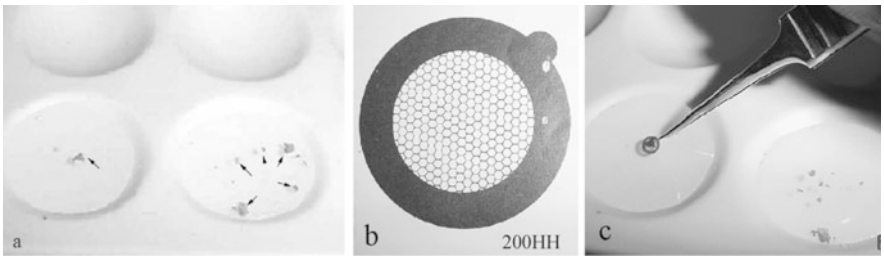


Fig. 5.19 Different steps of cleaning the replica. (**a**) Washing the replica; small *arrows* show the replicas. (**b**) Hexagonal tabbed grid. (**c**) Picking up the replica

Dissolution is followed by several rinses with distilled water. When organic solvents are used, care must be taken to prevent strong transitions between solutions with very different surface tensions, instead moving between baths of decreasing concentrations, ending with baths of pure water.

The replica is transferred between these different baths using a platinum loop that resists the different chemical agents. This is the second delicate part of the technique.

The replica, which floats on the surface of the final rinse bath, is then picked up on a copper support grid with a 400 or 600 mesh, preferably a hexagonal mesh, which has a small tab for holding the grid with a fine tweezer (Figs. 5.19b, c).

The grid is deposited in a glass Petri dish lined with four to five layers of filter paper. The replica must be facing upward, not touching the filter paper. In order to eliminate the collodion layer, the filter paper is gently soaked with a collodion solvent (isoamyl acetate, ether, chloroform, or methanol). The box is hermetically sealed and left overnight. Finally, the replica is ready to be observed in the TEM.

Technique Involving Fracture Started with a Razor Blade

A sample of 2–4 mm^3 is frozen on the support of the device using one of the methods described in the physical fixation technique. Under liquid nitrogen cooling, the sample is transferred into the device‘s chamber. After temperature and vacuum stabilization, the sample‘s surface is planed down with successive passes of a razor blade. Then the fracture is started by the razor blade on one side of the sample. The fracture must spread on its own through the interior of the sample. Next, platinum shadowing is performed, with a 45° angle, and then carbon coating at 90°. The sample is returned to room temperature and the replica is picked up as in the previous example. For this technique, protection with a collodion layer is not necessary.

4.2.3 Observations

In both cases, TEM observation must be done at a voltage of 80 KeV. It requires patience and a great deal of experience to find the replica that contains the fractured sample.

4.3 Variants

For molecule-sized samples, after fracturing the frozen suspension, it is possible to evaporate the ice from the sample surface in order to increase relief: This is cryo-sublimation (see "Methodology", Chapter 4). This is carried out in the cooled chamber; the sample is reheated to around 153 K while the cylinder surrounding the specimen holder is cooled with liquid nitrogen. The vacuum extracts the ice from the sample, which is trapped on the extremely cold cylinder surface. The time required for this operation can be between a few minutes and a few hours depending on the result needed.

4.4 Advantages

This is the only technique that can be used to observe samples in their hydrated state without deformations or displacement due to the processes involved in chemical preparations. The shadowing makes it possible to get a 3D view of morphology. It is used to measure particle size (diameter or height) (see "Methodology", Chapter 4, Section 7 and "Techniques", Chapter 7, Section 1). It is very useful when investigating nanospheres, their walls, and their contents (Figs. 5.20 and 5.21). The double-cupel technique is used to make two opposite replicas enabling the observation of the relief (concave and convex) of the same structure. The shadowing direction makes it possible to determine whether the structure is convex or concave.

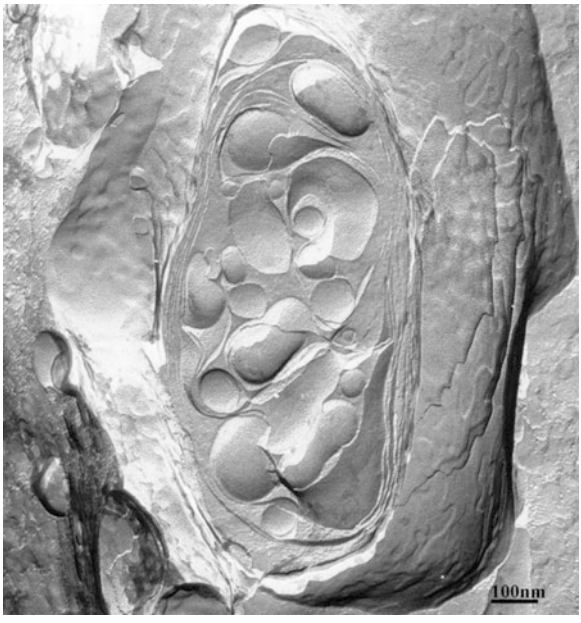


Fig. 5.20 Liposomes enclosed in a multilayer envelope (*J. Boumendil, CMEABG, UCB-Lyon 1*)

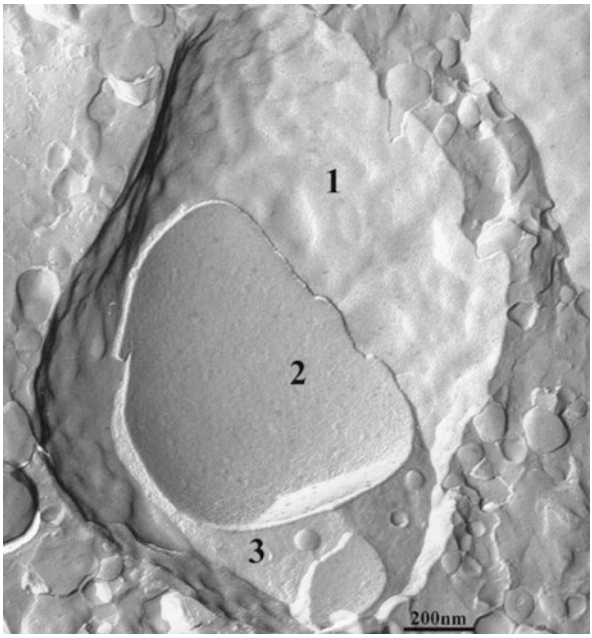


Fig. 5.21 Monolayer liposome of which we can see the outer face (1) and the inner face (3). It is enclosed in a globule (2) of an active substance. (*J. Boumendil, CMEABG, UCB-Lyon 1*)

In hydrated biological materials, the fracture is always produced in the area of least resistance, which is the double phospholipid layer of the membranes.

This technique enables observation inside the intercellular junctions, membranes, and nuclear pores. It also helps to highlight the protein particles present in the membranes and to see their arrangement (Figs. 5.22 and 5.23).

4.5 Limitations

The replica is limited to topography observation and produces a reverse image of the sample surface, making interpretation difficult. The replica no longer contains the sample, but only a mold of its surface in the fracture area, which rules out the possibility of performing chemical analysis. The sample′s fracture area is random in the bulk sample. It must be kept in mind that a replica is often more or less folded and contains areas composed only of pure carbon film or areas of the replica of the cupel. Sample dissolution is not always complete or, worse, the sample does not fracture in the area of interest. During TEM observation, it is often difficult to find the large areas, representing the fractured sample. It is strongly recommended to perform the technique at least twice for the same type of material in order to confirm the result.

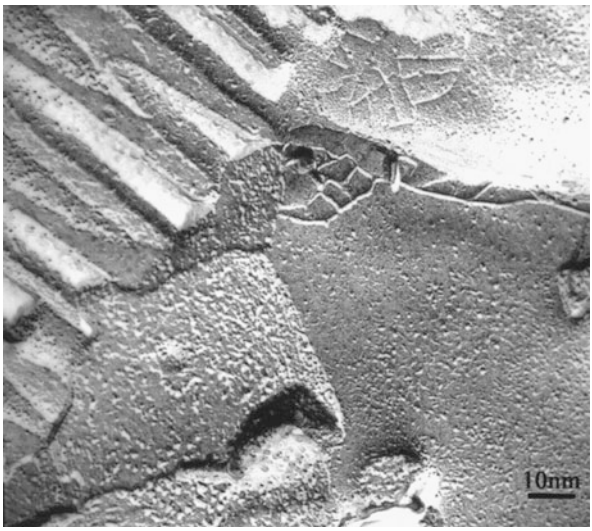


Fig. 5.22 Junction locked between two duodenum cells, microvillosities in *upper left-hand* corner (*J. Boumendil, CMEABG, UCB-Lyon 1*)

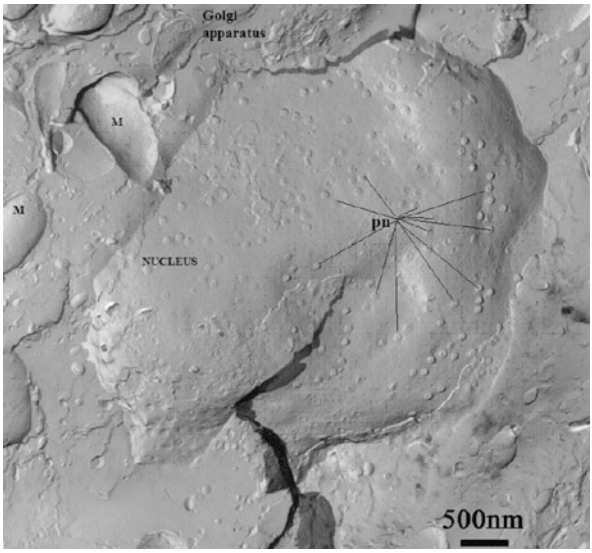


Fig. 5.23 Nucleus with its nuclear pores (pn) surrounded by mitochondria (M), vacuoles, and a small piece of the Golgi apparatus (*J. Boumendil, CMEABG, UCB-Lyon 1*)

Proper performance of the technique requires the knowledge of chemicals capable of dissolving the samples without damage, and it is often difficult to find the right combination. Expensive equipment is needed. Procedures are time consuming; between 1 and 3 days might be needed in order to get a good replica.

4.6 Artifacts

The freezing technique can result in the formation of ice crystals, leading to morphology deformations and phase segregations (Fig. 5.24). It can result in a loss of microstructure. A partially dissolved sample can lead to false interpretations. The replica is very fragile when handled and often fractures during the manipulations (Fig. 5.25). It can even explode during cleaning, a phenomenon caused by the surface tension of the solutions used. Thus, the technique must be started again.

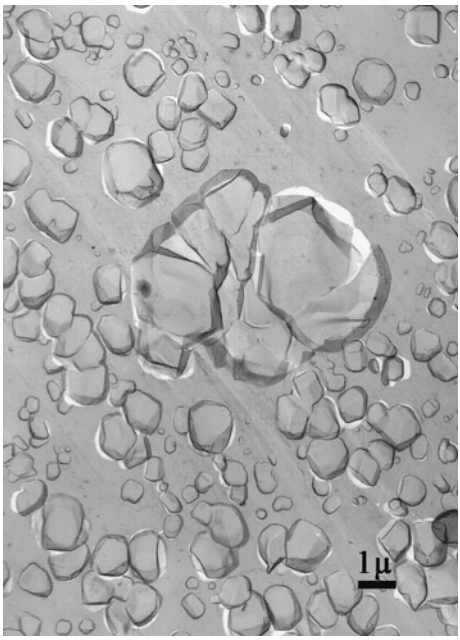


Fig. 5.24 Example of cubic ice crystals in a replica (*J. Boumendil, CMEABG UCB-Lyon 1; Bernard Lyon 1*)

4.7 Type of Analysis

Topography.

4.8 Risks

There is a risk of burns or anoxemia related to the use of liquid nitrogen, risks of corrosion and burns caused by the strong acids. There can be risks related to the toxic or carcinogenic characteristics of the solvents used (benzene, toluene, etc.). This

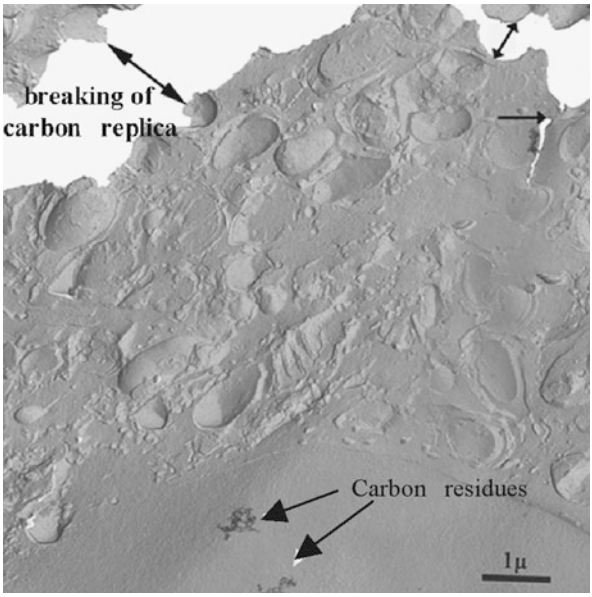


Fig. 5.25 Breaking at the top of the replica and the presence of carbon residues (*J. Boumendil, CMEABG, Université; Cl. Bernard Lyon 1*)

method requires working under a fume hood, with special protective gear (gloves, boots, and goggles).

4.9 Conclusion

The technique is routinely used to determine the size and distribution of fine particles in suspension. It is used to observe complex structures in their hydrated state, more or less polymerized, such as fibers and latexes. It highlights the internal morphology of bulk materials without the need for fixation, dehydration, embedding, or ultramicrotomy. Furthermore, through the same fracturing principle, it can be used to see the inside of organic membranes at the double phospholipidic layer. It is a supplement to the negative-staining contrast or film of frozen suspension techniques.

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Chapter 6
Techniques Specific to Fine Particles

1 Dispersion of Fine Particle Materials

1.1 Principle

Dispersion of fine particle materials is used to isolate fine particles onto a support grid film so that they can be observed under the microscope.

It consists of dispersing fine particles of a material. Generally, the material is suspended in various liquids, followed by stirring, in order to improve dispersion of the particles that are then deposited onto the film of a support grid (see the techniques on continuous or holey support films in Chapter 2, Sections 13 and 14).

This technique, which is easy to use, can be applied to any type of fine particles whether they are micrometric or nanometric in size.

1.2 Operating Mode

1.2.1 Equipment and Supplies

Usually a mechanical, magnetic, or ultrasound stirrer is used to disperse the particles. A micropipette can be useful for precise dosing when depositing the suspension containing the fine particles onto a support grid holding a continuous or holey film (see the techniques on continuous or holey support films).

1.2.2 Procedure

Fine particles are suspended in a volatile liquid adapted to the specificities of the material. The solvent used must be compatible with the material; usually demineralized, distilled, or even twice-distilled water is used for minerals, ceramics, and biological materials. In some cases, when there is a risk of interaction with water, other solvents are used, e.g., a buffer solution for biological materials undergoing immunological processing. In other cases, alcohol is used because of its rapid evaporation; this is the case for catalysts and nanomaterials in general, which tend to re-agglomerate during the overly slow evaporation of the drop of water. And

J. Ayache et al., *Sample Preparation Handbook for Transmission Electron Microscopy*,
DOI 10.1007/978-1-4419-5975-1_6,

lastly, hydrocarbons are used to investigate petroleum or other heavy carbonaceous materials containing organic matter.

The suspension is agitated using a mechanical, magnetic, or ultrasound stirrer, in order to separate the agglomerated particles and make the solution homogeneous. Attention must be paid to not to destroy the particles during agitation, in particular nanospheres and biological samples (e.g., viruses). In these cases, dilution is often necessary in order to have a very low material concentration, so that the particles are sufficiently isolated from one another on the support film. Immediately after agitation, a drop of the suspension is sampled using a micropipette or a simple Pasteur pipette, and the drop is placed on a film on a support grid. The preparation is ready after the water or solvent chosen has completely evaporated.

When water is used as dispersion liquid, drying (which can be as long as a few hours) is accelerated by placing the support grid in an oven or under an infrared lamp. Drying can shorten the time the water droplet takes to dry on the support grid and alleviate the related drawbacks. In fact, three concomitant phenomena occur as the diameter of the droplet decreases: increasing surface tension tends to agglomerate the particles; particles tend to stick to one another by flocculation (Figs. 6.1, 6.2; and 6.3) the support film tends to fix particles to its surface via the electric potential effect of the film, when its ionic charge is opposite to that of the particles themselves. Rapid drying helps to limit the artificial formation of aggregates. However, the drying process must be gentle because otherwise there is a risk of breaking the support grid film. In the case of samples composed of light atoms (polymers and biological materials), applying a negative stain before the droplet completely dries can prevent these drawbacks (Chapter 7, Section 2).

The use of alcohol, instead of water, partially eliminates these drawbacks. When the drop of alcohol suspension is placed on the support grid, the drop immediately spreads out and the alcohol evaporates very quickly; the particles do not have time

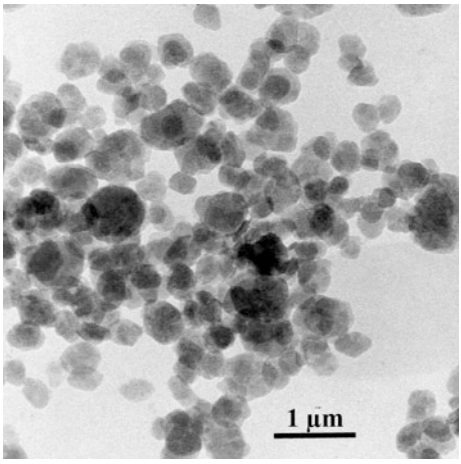

Fig. 6.1 Illite clay. Particles dispersed in distilled water under ultrasound and deposited on a continuous film. Too high a concentration creates aggregates and hinders observation (*J. Duplay, EOST, Strasbourg*)

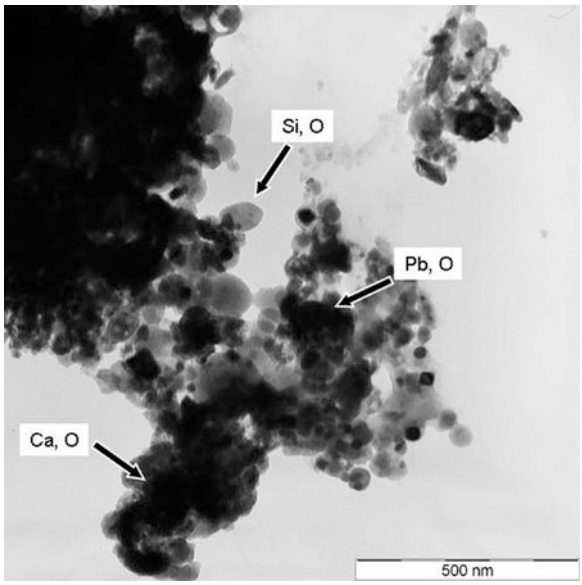

Fig. 6.2 TEM image of grains of dust collected on a filter and washed with water. EDS analysis is possible despite the aggregation of the particles (*J. Boumendil, Université Claude Bernard-Lyon 1*)

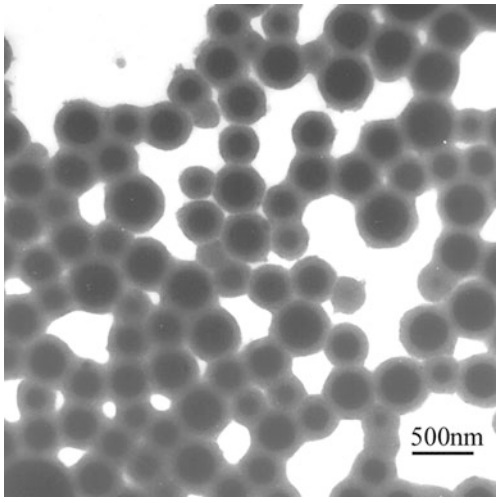

Fig. 6.3 Core–shell: Polymer beads with a core and shell made of different polymers. Wetting agent residues make the beads stick to one another (*J. Boumendil, Université Claude Bernard-Lyon 1*)

to aggregate. *Caution*: The collodion support on the grid is destroyed by alcohol. In this case, a support grid covered with Formvar or carbon should be used.

Films can be processed by ionization in order to promote particle fixation onto the support grids and to prevent aggregates (Chapter 2, Sections 13 and 14).

For some materials, suitable dispersion agents can be used, but they are often poorly eliminated and hinder observation (Fig. 6.3).

1.3 Variants

1.3.1 Dry Dispersion

In the case of an ultrafine powder, samples are taken directly by contact using a support grid carrying a film. Excess powder that does not adhere to the grid is eliminated by lightly tapping the grid. This is the case when preparing nanomaterials, e.g., carbon nanotubes (Figs. 6.4, 6.5, and 6.6). The support grid carrying a holey film is held at the tip of a pair of tweezers, and the side of the film with the fine particles is delicately rubbed on a glass slide. The particles adhere by contact onto the film. The procedure is simple but delicate, because the film will break if rubbed too hard. Excess particles that do not adhere to the film must absolutely be removed in order to prevent the powder from contaminating the microscope.

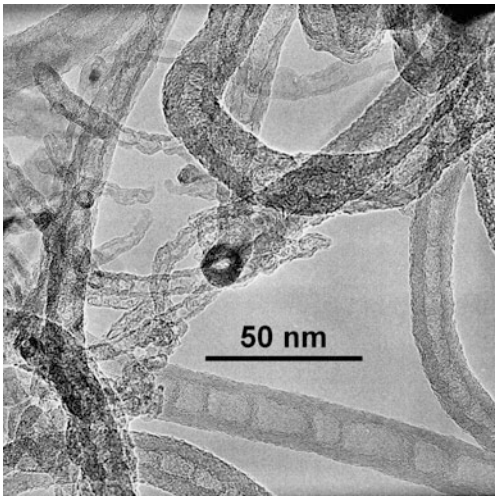

Fig. 6.4 Carbon nanotubes. Dry dispersion of particles on a holey film (*C.S. Cojocaru, IPCMS, Strasbourg*)

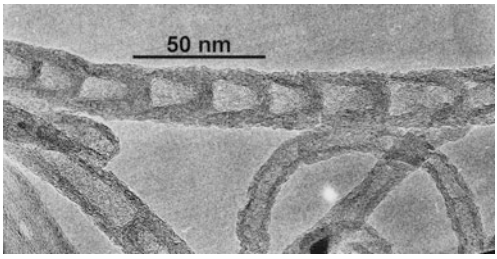

Fig. 6.5 Details on the internal structure of a carbon nanotube (*C.S. Cojocaru, IPCMS, Strasbourg*)

1.3.2 Dispersion on the Surface of Water (Langmuir Film)

The powder is suspended in alcohol. A drop of this suspension is placed on the surface of a crystallizing dish filled with water. An extremely thin film of fine particles forms, which is recovered on a support grid coated with a carbon film. In particular, this technique is used for clay-type minerals that are in the form of platelets

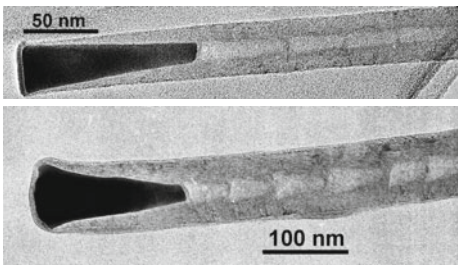

Fig. 6.6 Detail of the ends of carbon nanotubes plugged with a metallic nickel particle when they were produced (*C.S. Cojocaru, IPCMS, Strasbourg*)

(small plates). The surface tension of the water will spread the flakes out, constituting the small-particle platelets, and the resulting film will be a single layer of flakes. Placing a support grid held at the tip of a pair of tweezers, with the film side in contact with the surface of water in the crystallizer, is enough to recover the particles by capillary action. The grid is then quickly dried before its observation under the microscope.

1.3.3 Aerosol Dispersion

When particles are suspended in a gas, e.g., dust floating in the atmosphere, a film-coated support grid just needs to be exposed to a gaseous flow carrying the particles. Grid exposure time is chosen so as to get a suitable concentration of dust on the preparation (Fig. 6.2).

This technique can be used to detect asbestos in a room, as well as to quantify the number of asbestos fibers per liter of filtered air (or to check atmospheric pollution). In this case, the suction of the building's air pumping system over a very long period of time (generally 1 week) is used and the particles are collected on a filter. This filter is then burned, and the asbestos particles are suspended in a known quantity of water and a calibrated drop is placed on a support grid film. Statistical investigation is used to identify and quantify the asbestos fibers contained in the room (Figs. 6.7 and 6.8).

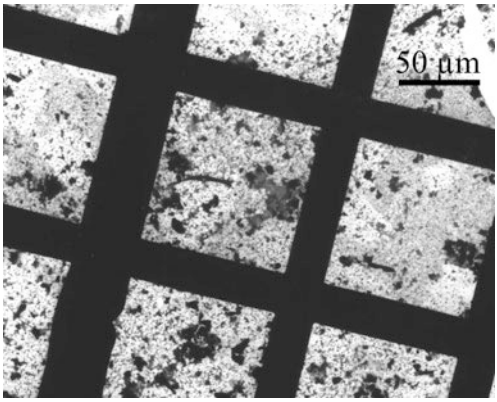

Fig. 6.7 Asbestos fibers dispersed on a carbon film. TEM image at very low magnification showing homogeneous distribution. The support grid mesh measures 100 µm (*G. Ehret, IPCMS, Strasbourg*)

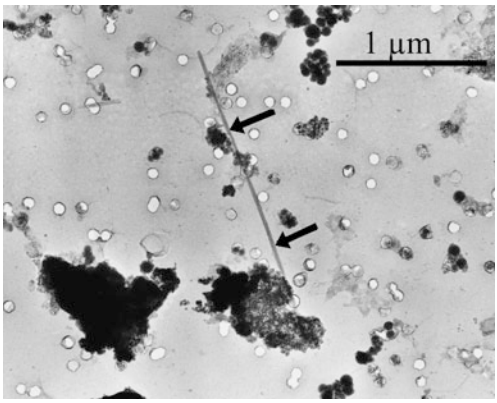

Fig. 6.8 High-magnification image of a chrysotile asbestos particle (indicated by the *arrows*) (*G. Ehret, IPCMS, Strasbourg*)

1.3.4 "Spin Coating" Dispersion

"Spin coating" dispersion is used to disperse particles, while avoiding the successive dilution steps that are often necessary in order to obtain an adequate quantity of particles, i.e., not agglomerated. This technique involves a rotating motorized support (Fig. 6.9) with variable speed, on which a TEM support grid coated with a carbon film or a support for SEM observation can be attached. The rotating support is spun and a drop of suspension is placed on the TEM support grid (or SEM support). The rotation allows the suspension droplet, and thus the fine particle material, to be spread uniformly.

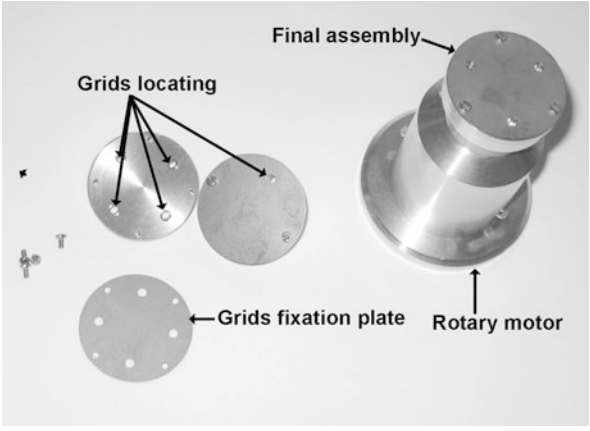

Fig. 6.9 Device and tools for performing the spin-coating-dispersion technique

1.3.5 Dispersion and Spreading Nucleic Acids (DNA, RNA, and Associated Proteins)

There are three spreading methods.

Cytochrome *c* Method

Cytochrome *c* is a basic protein that forms a thin film on the surface of water. When cytochrome *c* is mixed with a nucleic acid, it creates a complex that locks the nucleic acid into the mesh of the film and preserves the spatial conformation of the nucleic acid. This is a classic method for viewing double-chain DNA molecules and DNA–protein complexes. It is necessary to use dispersion agents such as urea or formamide instead so that the single-chain DNA spreads out without forming aggregates. Chemical fixation with aldehydes may be necessary for stabilizing DNA–protein complexes that can dissociate during spreading.

– The film is recovered from the surface of the water in a crystallizing dish according to the Kleinschmidt technique (Fig. 6.10). The film is recovered using a grid covered with collodion carbon. The grid with the cytochrome and DNA film is placed in contact with pure alcohol, which will eliminate the water and stabilize

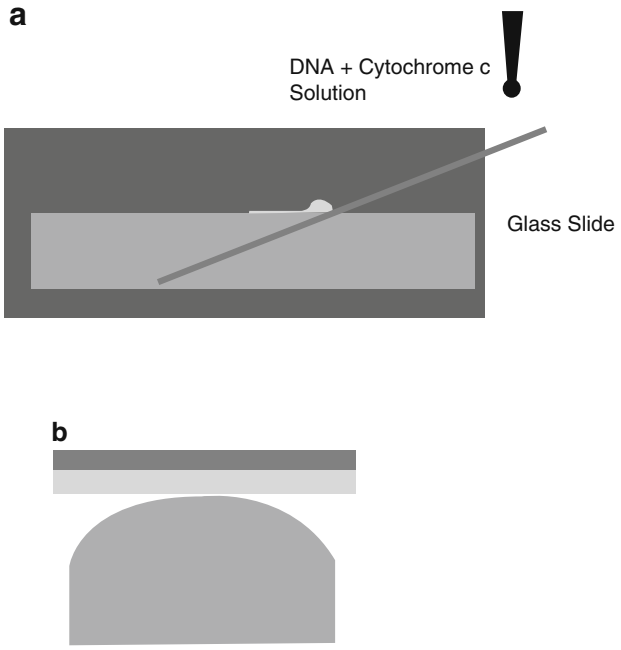

Fig. 6.10 **a** Kleinschmidt method for spreading DNA with cytochrome *c*. **b** Washing with a drop of ethanol

the film. This technique requires a fairly large quantity of cytochrome–nucleic acid solution, which is often hard to obtain.

– In a second method, the film can be recovered on the surface of a 1 ml drop of water (Fig. 6.11) by sliding a microdrop of cytochrome *c*–nucleic acid mixture along a 2-mm-diameter glass rod. The film is recovered by placing a grid covered with collodion carbon over it: This is called the Inman method (Fig. 6.11), which has widely been used to view partially denatured DNA molecules. Formamide is then essential for stabilizing the single-chain segments corresponding to the most easily denatured segments because they are rich in adenine–thymine.
– A third method, the diffusion method (Fig. 6.12), can also be used. This method consists of placing a drop of the cytochrome *c* nucleic acid mixture onto a piece of adhesive tape. The molecule film rises to the surface of the drop and can be recovered on a grid covered with collodion carbon. This method is used to work with small volumes of DNA cytochrome *c* solution.

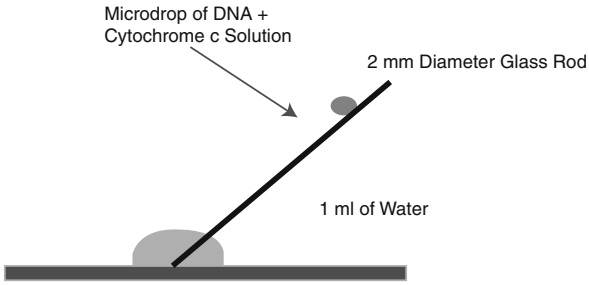

Fig. 6.11 Inman's method for spreading DNA with cytochrome *c*

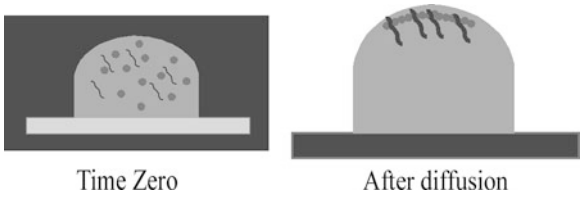

Fig. 6.12 Diffusion spreading method for DNA with cytochrome *c*

It may be necessary to use a glow discharge to treat the carbon films coating the grids in order to improve their wettability.

Detergent Method

This method is more specific to investigating single-strand or double-strand DNA with DNA–protein complexes. These complexes must be stabilized with an aldehyde pre-fixation.

Detergent is used in place of cytochrome *c* to create the film. The detergent used is benzyl alkyl ammonium chloride (BAC). It may be necessary to fix the nucleic acids with aldehydes before placing them into contact with the detergent.

Adsorption Method

There are three different procedures that can be used:

1. Deposit a polylysine spread by "spin coating," which serves as a glue between the carbon film of the grid and the spread-out molecules.
2. Use divalent cations such as Mg^{++} added to the DNA solution to facilitate binding to the grid. This method risks changing the spatial conformation of the molecules. This procedure is widely used to immobilize molecules on mica slides in order to observe them using atomic force microscopy (AFM).
3. Use the Dubochet method, which consists of depositing electrical charges on the surface of the carbon film obtained with a pentylamine plasma. Vaporizing pentylamine will deposit NH^{+} ions, which will bind to the DNA molecules that are negatively charged. The use of an amylamine vapor will make it possible to deposit negative charges.

The lack of contrast between these spread-out molecules next requires a negative or positive contrast or shadowing (Figs. 6.13 and 6.14).

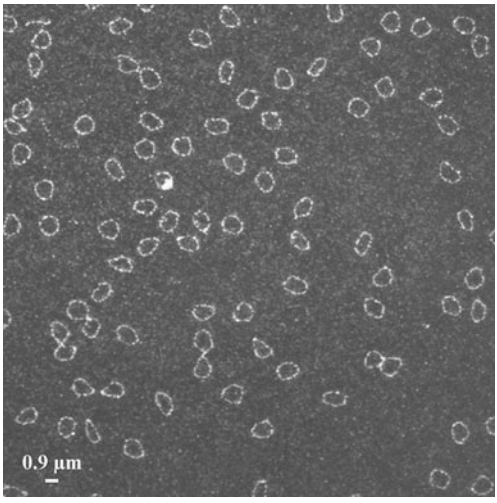

Fig. 6.13 Dark-field image of DNA *minicircles* deposited using the Dubochet method and positive staining (*E. Le Cam, CNRS-UMR8126-IGR, Villejuif*)

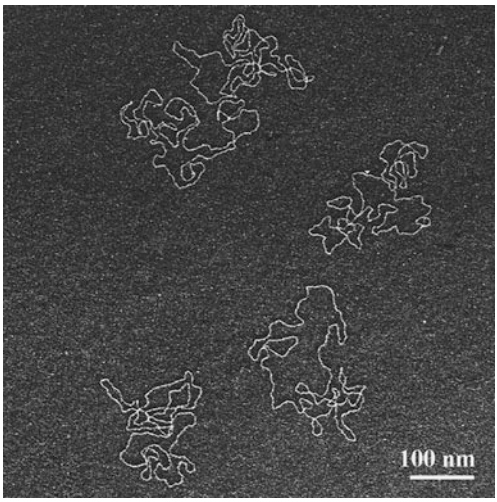

Fig. 6.14 Dark-field image of DNA plasmid deposited using the Dubochet method and positive staining combined with shadowing techniques (*E. Delain, CNRS-UMR8126-IGR, Villejuif*)

1.4 Advantages

This is a very rapid technique. It enables the statistical analysis of fine particles. Knowing the dilution of the suspension and the volume deposited on the grid enables us to increase the amount of initial material. This is the case for the determination and quantification of asbestos fibers in the atmosphere of a room (Fig. 6.8), in order to measure the concentration of viral particles in a vaccine, etc.

1.5 Limitations

The presence of certain additives, such as dispersion agents, can hinder the technique (Fig. 6.3).

1.6 Artifacts

This technique can lead to morphology alteration during desiccation for fragile materials in liquid solution (biological materials, polymers). We can often remedy this by chemical or physical fixation (see Chapter 6, Section 2).

Observation will be hindered when the particle concentration is too high or if agglomerates or dispersion residues are present in the sample (Figs. 6.1 and 6.3).

1.7 Type of Analysis

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

1.8 Risks

The only risk is inhalation of noxious aerosols.

1.9 Conclusion

This is the simplest and fastest technique for fine particle materials.

This technique is particularly suitable for determining concentrations of asbestos fibers, polymers, viruses, etc.

In the case of materials with a low electronic density, the negative-staining and positive-staining contrast techniques can improve observation.

The frozen suspension technique is required for highly brittle nanomaterials.

This technique complements the freeze fracture or ultramicrotomy techniques.

2 Frozen Hydrated Film of Single Particles

2.1 Principle

This technique is based on the direct observation of a thin film of vitreous ice containing fine particles of a biological material in its native environment, i.e., without chemical fixation (DNA, proteins, DNA–protein complexes, viruses, polymers, etc.).

The method consists of performing a physical fixation of a small volume via the ultrarapid freezing of a drop of water (or freezable solvent) deposited on a microscope grid coated with a holey carbon film. Vitrification of the aqueous phase into an amorphous solid phase without crystallization stabilizes heavily hydrated biological structures.

This technique is applied to small-sized fine particles dispersed in liquid solution whose components are composed of light elements (C, O, H, N, etc.). It is widely used for investigating polymers and biological materials. It requires cold TEM observation, i.e., cryomicroscopy. It is often combined with 3D reconstruction of complex molecular systems. This technique can also be suitable for some mixed–composite fine particles.

2.2 Operating Mode

2.2.1 Equipment and Supplies

The ultrarapid freezing equipment used is called a cryo-plunger (Fig. 6.15). It is composed of a cryogenic Dewar (ethane, propane, etc.) filled with liquid nitrogen cooled to 98 K and a system for plunging the TEM grid into the cryogen. The plunger system is used to plunge a clamp holding the support grid and the sample into the cryogenic reservoir. A chamber can be used to control temperature and hydration. These systems are commercially available.

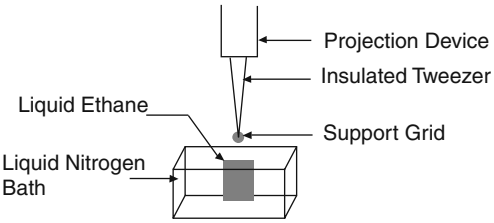

Fig. 6.15 Cryo-plunger diagram, device for freezing with ethane

2.2.2 Procedure

The procedure involves several steps, each of which is very strict, in order to produce an electron-transparent thin vitrified film in a reproducible manner.

Preparing to Deposit the Suspension on the TEM Support Grid

A 4–5 μl drop of an aqueous suspension of previously dispersed particles is spread on a copper grid (300 or 400 mesh), 3 mm in diameter, coated with a holey carbon film (Fig. 6.16).

The concentration of these particles must be adjusted in order to be able to obtain a vitreous ice film with a homogeneous distribution of the particles inside the holes of the carbon film.

Holey films must be thick enough to be resistant and thin enough to provide a layer of ice that "coats" the objects as well as allows for their observation in all possible orientations. The film is usually thinner in the center of the holes. The average size of the holes must be 1–3 μm.

In order to make a proper deposit of the suspension, the holey film must also be sufficiently hydrophilic (polar) so that the drop of suspension moistens the whole grid. Grid wettability can be improved either by first plunging them in ethyl acetate or by treating the grids using an electrical discharge (see Chapter 2, Sections 13 and 14).

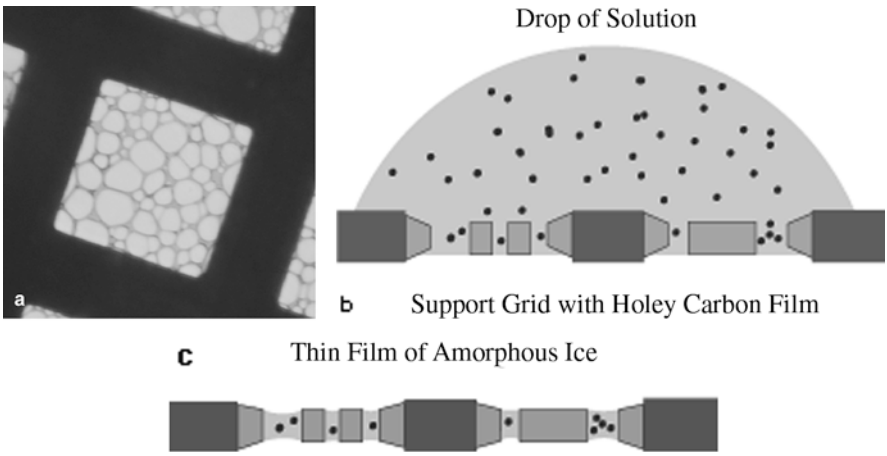

Fig. 6.16 **a** TEM image of a holey carbon support grid; diagram of the particle suspension **b** before and **c** after sponging the drop. The particle suspension can be seen in the holes of the carbon film

Preparation of the Frozen Hydrated Film

The grid holding the drop of particle suspension to be frozen is held between the jaws of a pair of ultrathin tweezers mounted on the plunger. Before cryofixation, excess solution must be removed from the grid in order to leave behind a very small film of the suspension only in the holes of the carbon membrane. To do so, the grid is delicately and quickly blotted using an ashless filter paper placed parallel to the drop of solution on the grid. This step is called "grid blotting" and is the determinant for obtaining a frozen hydrated film of the right thickness in a maximum number of holes in the carbon membrane. This is done either manually by using a single ashless filter paper or mechanically by pressing the grid between two ashless filter papers. Immediately after blotting, the plunger is released. A manual release cryo-plunger is represented in Fig. 6.17. This manual technique has the advantage of being simple and inexpensive, with the drawback of being difficult to implement. The mechanical method using a cryo-plunger device represents an improvement in routinely producing a thin film of ice. Throughout the preparation, humidity and temperature must be monitored so as to prevent excessive dehydration of the suspension drop. Nevertheless, the experimental conditions to obtain a good reproducibility of the film thickness remain difficult to achieve.

Ultrarapid Freezing

This is freezing by projection plunging. When the plunger is released, the suspension of particles or macromolecules remaining on the grid is plunged into the cooling bath and transformed into an amorphous thin frozen hydrated film. This

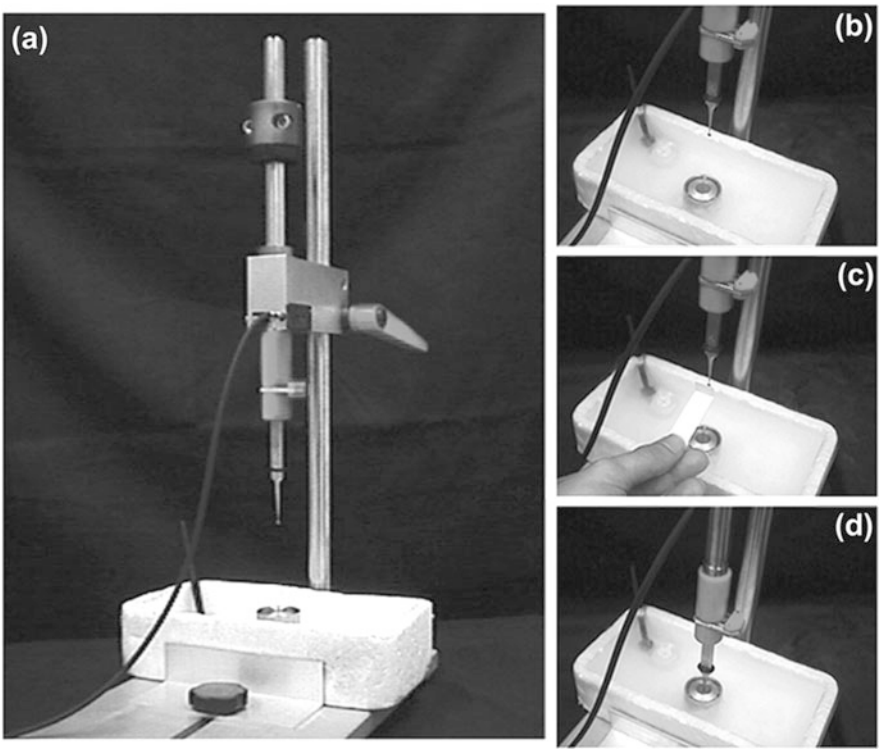

Fig. 6.17 **a** Cryo-plunger and reservoir cooled by a bath of liquid nitrogen; **b, c, d** show the three steps before plunging: **b** placing the grid on the plunger, **c** blotting, and **d** ultrarapid cryofixation in ethane

amorphous ice immobilizes the floating molecules of the solution. After a few seconds, the cryofixed grid is removed from the ethane bath, then transferred into the cold dry vapors of liquid nitrogen in the support for the grid storage, and placed in the liquid nitrogen bath of the cryo-plunger. This support can contain several grids. It is then transferred into the nitrogen vapors, which are either in a nitrogen reservoir or in the cryo-transfer stage of the TEM cryo-specimen holder for observation.

Cryo-Transfer

A second cryo-transfer is carried out to move the sample storage box from the cryo-plunger to the cryo-transfer stage of the cooled TEM specimen holder. This cryo-transfer stage, shown in Fig. 6.18a, includes a chamber cooled with a bath of liquid nitrogen (background) and the cold specimen holder (foreground), which can be inserted into this chamber. Note that at the right end of the specimen holder, there is a Dewar filled with liquid nitrogen that will cool the specimen holder located at the

other end of the shaft. Figure 6.18b shows the details of the chamber in which the grid is transferred from the transport reservoir to the cooled TEM specimen holder. This final step must be performed using a pair of tweezers whose tips are first cooled in liquid nitrogen in order to prevent any change in temperature that would cause the formation of ice crystals in the preparation.

Fig. 6.18 **a** Cryo-transfer stage: The cooled chamber is in the background, and the cold TEM specimen holder is in the foreground. **b** Cooled chamber of the TEM specimen holder for transferring the grid

2.2.3 Observation in Cryo-microscopy

Observation in bright-field mode, which is mandatory in cold conditions, must be performed with a minimum dose of electrons so as not to change the vitreous ice (electron dose < 1,000 e/nm^2/s). This is performed in low-dose mode (a special mode on the microscope). Figure 6.19 shows a bright-field cryo-microscopy TEM image of a frozen hydrated film of a tomato bushy stunt virus (TBSV) in two different conformations.

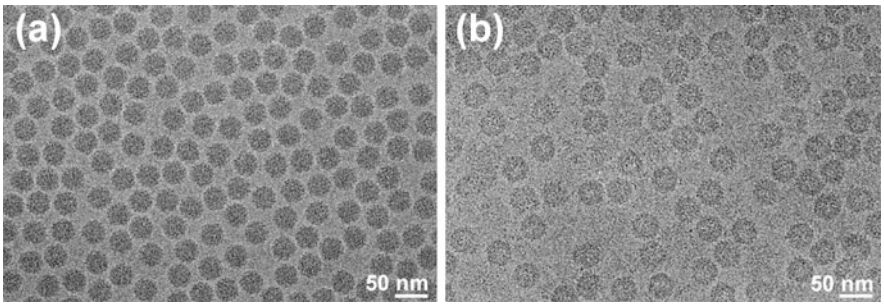

Fig. 6.19 TEM image of a frozen hydrated film of a TBSV virus in two different conformations (*E. Larquet et N. Boisset, IMPMC-UMR CNRS-Université, Paris*)

2.3 Variants

2.3.1 Cryo-plunger in a Controlled Atmosphere Chamber

A new cryo-plunger device developed by F. Livolant and A. Leforestier is supplemented by a chamber allowing for the control of the temperature and humidity and a mechanical system for controlling pressure and sample blotting time. Controlling experimental conditions during cryofixation helps to maintain the ionic concentration of the particles in solution; maintain the temperature in order to prevent evaporation, which ensures the proper preservation of protein conformation, notably thermo-sensitive proteins; and systematically reproduce a frozen hydrated film of particles in suspension, with the same thickness (Fig. 6.20).

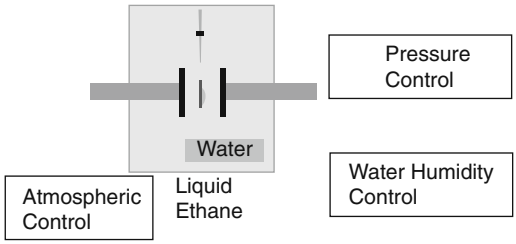

Fig. 6.20 Cryo-plunger in a controlled-atmosphere chamber for controlling temperature and humidity and a mechanical system for controlling blotting time and pressure

2.3.2 Cryo-fixation of a Contrasted Sample

The sample can be contrasted by adding a contrastant, e.g., phosphotungstic acid. However, this method is only applied as a last resort when it is not possible to see the objects in the ice. It helps to preserve the hydrated state but the specimen is no longer native. This type of preparation is called "cryo-negative staining" (Adrian et al., 1998).

2.4 Advantages

As there is no carbon film in the observation areas, nor chemical preparation (addition of fixatives and/or contrastants) or desiccation, it is possible to observe the sample in its native hydrated state. This technique is used to obtain the best resolution for single-particle microscopy analysis.

Using images of several thousand specimens and with the help of image analysis software, it is possible to make 3D reconstructions of specimens and investigate the spatial conformations of complex molecular systems such as viruses (Fig. 6.19) or macromolecules such as proteins.

2.5 Limitations

This technique can be applied only to nanometric-sized single particles in suspension. Surface tension forces on the thin film of water exert an influence on the distribution of molecules but also exert stresses on large objects.

The preparation is very sensitive to the electron beam. Manipulation and successful preparation require a great deal of practice. Producing a very thin film of vitreous ice is difficult. The technique is expensive and requires special equipment on the microscope (cooled stage and transfer system).

2.6 Artifacts

Artifacts usually result from poor freezing. Ice crystals (Fig. 6.21) can form or the sample temperature rises at each transfer step, making observation impossible (Fig. 6.22). If plunging is not fast enough after blotting, the sample might start to desiccate, thus changing the structures or cryofixation. The structures to be observed may also change insidiously during observation under the effect of electron irradiation.

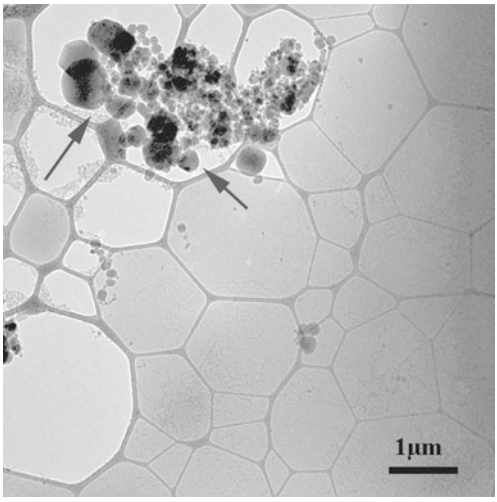

Fig. 6.21 Bright-field TEM image of a frozen suspension film presenting crystals of cubic and hexagonal ice on the carbon holes (*arrows*) without the frozen suspension film (*G. Pehau-Arnaudet, Institut Pasteur CNRS, Paris*)

2.7 Type of Analysis

Topography and structure.

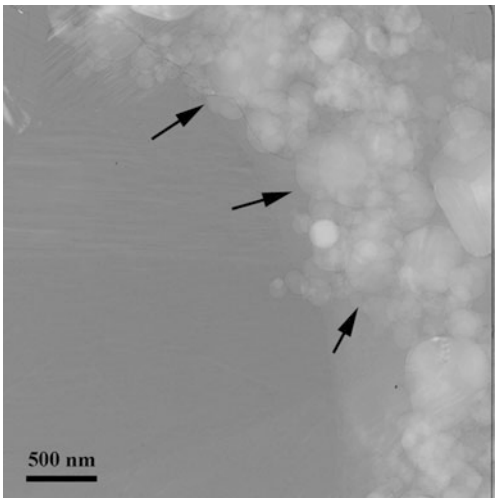

Fig. 6.22 Bright-field TEM image of a frozen suspension film showing the effects of improper freezing. Clusters of segregated crystals (*arrows*) on the edges of the holes of the carbon film can be seen (*S. Baconnais, CNRS-UMR8126-IGR, Villejuif*)

2.8 Risks

There are burn and explosion risks related to the use of liquid nitrogen and ethane gas.

2.9 Conclusion

The observation of a frozen hydrated film of a single particle suspension after cryofixation is the technique used to maintain the best preservation of biological material. It is a very useful technique for fine particle samples of very small sizes, such as single particles that give a low contrast. It is indispensable for samples that are particularly sensitive to desiccation. It complements the simplest negative-staining and decoration-shadowing techniques.

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Chapter 7
Contrast Enhancement and Labeling Techniques

1 Decoration Shadowing

1.1 Principle

This technique is used to enhance contrast in a sample. It is particularly useful for highlighting very faint surface relief. The method consists of depositing heavy-metal (high *Z*) particles on or around a sample's relief. The contrast of a sample is enhanced by shadowing resulting from a metallic deposit.

A very thin deposit is made by evaporating metals such as platinum, gold, chromium, or tungsten.

The technique is applied to materials comprising light elements, fine particles, or single layers, and, in particular, polymers, biological materials, and mixed–composite materials.

1.2 Operating Mode

1.2.1 Equipment and Supplies

The equipment generally consists of an evaporator under high vacuum containing at least one metal evaporation source and a tiltable specimen support stage. In order to get fine grains, it is recommended to use an evaporation gun and perform the operation in a clean vacuum (10^{-4} Pa) produced by a turbo-molecular pump.

1.2.2 Procedure

There are different procedures for the shadowing and decorating technique for fine particle or single-layer materials.

Unidirectional Shadowing Technique

This technique is applied to a material that is first dispersed onto a support grid covered with a carbon film. Shadowing is carried out with a fixed incidence angle

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DOI 10.1007/978-1-4419-5975-1_7,

between 30° and 45°, depending on the thickness of the structure to be highlighted (Fig. 7.1).

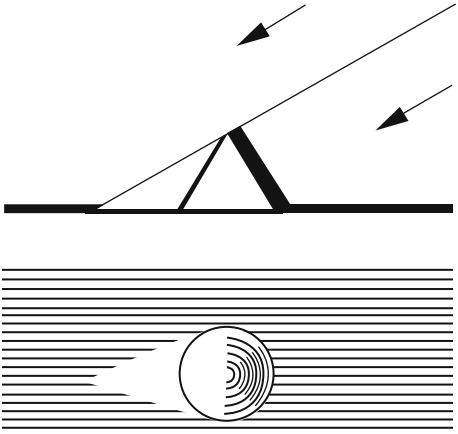

Fig. 7.1 Unidirectional direct shadowing

Unidirectional Shadowing Technique with Carbon Deposit Enhancement

The material is dispersed on a clean, flat surface with no roughness (freshly cleaved mica, polymer). Next, shadowing by metal evaporation under vacuum is performed at an angle of incidence between 5° and 45°. Shadowing is immediately followed by a carbon deposit at an angle of incidence of 90°, without interrupting the vacuum in the evaporation chamber, in order to form a continuous film. The entire set (sample/metallic shadowing particles/carbon film) is removed on the surface of water and recovered on a support grid.

In order for this technique to be reproducible on successive samples, the thickness of the deposited metal must be measured during deposition. Measurement can be merely visual, by estimating the level of grayness obtained either on a filter paper or a piece of porcelain placed next to the object. A quartz crystal can also be used to measure deposition thickness. The conductivity of quartz depends on the metallic layer covering it, but it also depends on the surrounding temperature. The temperature rise produced by metallic shadowing therefore disturbs the measurement, which is not truly valid unless we are working in a temperature-controlled cooled atmosphere. Thickness can be calculated based on the deposit intensity, duration, and distance between the source and the sample.

Both of the direct shadowing procedures are used to accentuate contrast and to measure the roughness of an object in the direction of the optical axis.

The choice of angle of incidence for the shadowing depends on the presumed height of the roughness of the sample relief.

Large angles, approximately 45°, are used for heavy relief.

Smaller angles between 5° and 30° are used for faint relief.

Directional shadowing is not used to highlight structures that are perpendicular to the shadowing direction. For example, pilis on a bacterium (Fig. 7.2) are not

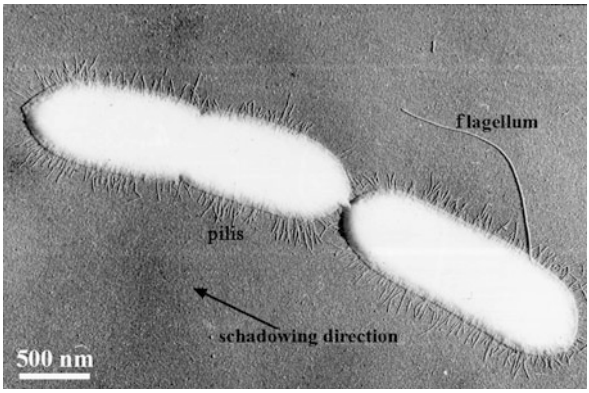

Fig. 7.2 *Escherichia coli* bacteria after direct unidirectional shadowing; the pilis at the ends are not well highlighted (*A. Ryter, Department Biologie moléculaire Institut Pasteur, Paris*)

visible at the summit because they are in the shadowing direction. This is why bidirectional shadowing (Fig. 7.8) is performed, or better still, rotary shadowing, which completely surrounds the structures (Fig. 7.9).

Decoration Techniques Using Markers

There are also different decoration procedures for bulk or thin layer materials involving the evaporation of "geometrical" markers in order to highlight their topography.

Decoration Using Gold

The marker is a spherical particle obtained by evaporating gold under high vacuum. Mobile gold atoms aggregate on the substrate, forming particles that fix themselves preferentially along the steps and/or local depressions on the surface of the sample. In the example below of a linear low-density polyethylene (ethylene–butene-1 copolymer, crystallinity rate $\tau_c = 40\%$), the gold particles are deposited at the interface of the crystalline and amorphous areas and form a double row. This is unlike linear polyethylene, ($\tau_c = 90\%$), where particles form a single row of gold grains.

For all of these samples, the amorphous areas are in depressions with regard to the surface of the crystallized areas. Furthermore, a ruthenium tetroxide dye can be used to localize the amorphous areas, which are darker in TEM bright-field images (Figs. 7.3 and 7.4).

Decoration Using Polymers

The marker is a non-isometric particle obtained by depositing a semi-crystalline polymer (generally polyethylene lamella). Polymer lamellae are not visible until after directional shadowing.

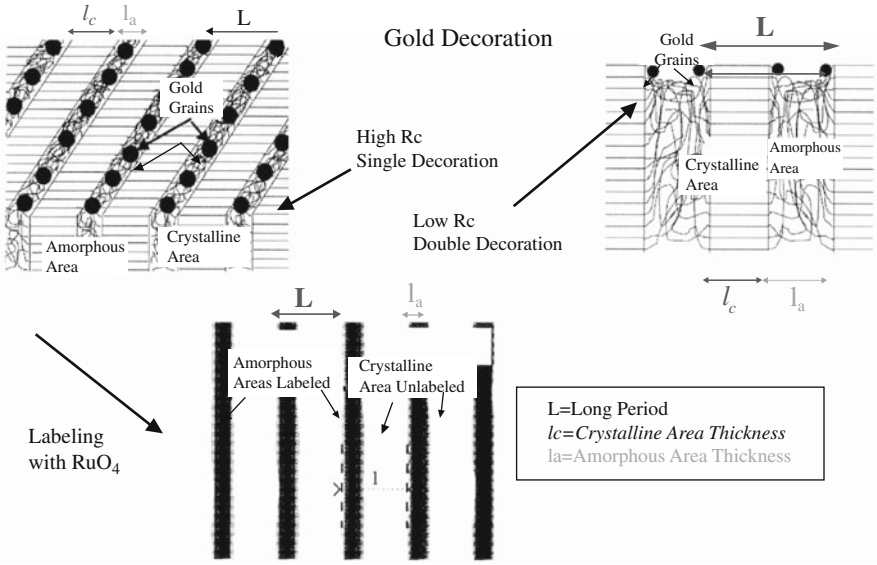

Fig. 7.3 Diagram illustrating decoration with gold

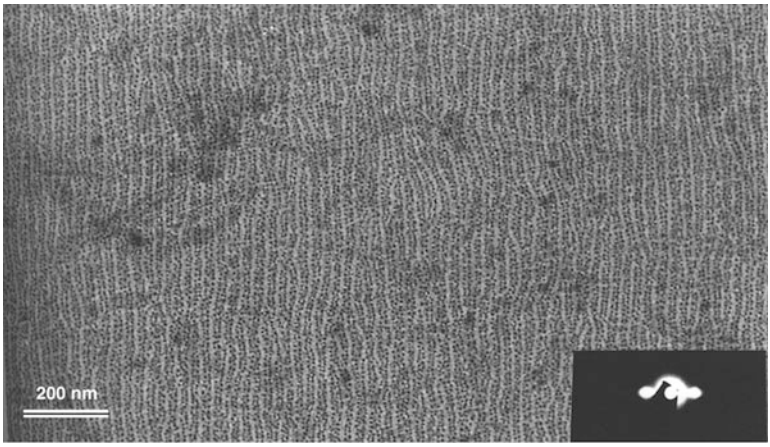

Fig. 7.4 Bright-field image of a linear low-density polyethylene film (ethylene–butene-1) epitaxially crystallized on a benzoic acid single crystal. The sample has been decorated with gold and dyed with ruthenium tetroxide. Ruthenium tetroxide is preferentially absorbed in the amorphous zones that show up as darker areas in the picture. *Inset*: Diffraction of the rows of gold particles at low Bragg angles (camera length 6.20 m) is used to measure the long period (L = total thickness, i.e., the thickness of the amorphous area (l_a) + crystalline area thickness (l_c). $L = 20$ nm. See Fig. 7.3) (*L. Loos, B.A. Lotz, A. Thierry, ICS, Strasbourg*)

In the example chosen, the surface of a polyethylene single crystal obtained by crystallization in diluted solution is decorated using crystalline lamellae of polyethylene deposited by vaporization under secondary vacuum (10^{-2} Pa) (Fig. 7.5). The

lamellae are perpendicular to the growth faces and help highlight the regular folds of the polymer chains in each growth sector (Figs. 7.6 and 7.7).

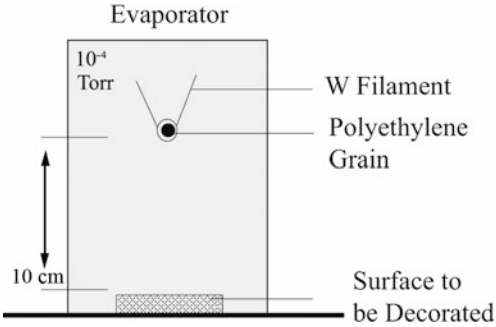

Fig. 7.5 Vaporization of polyethylene under vacuum

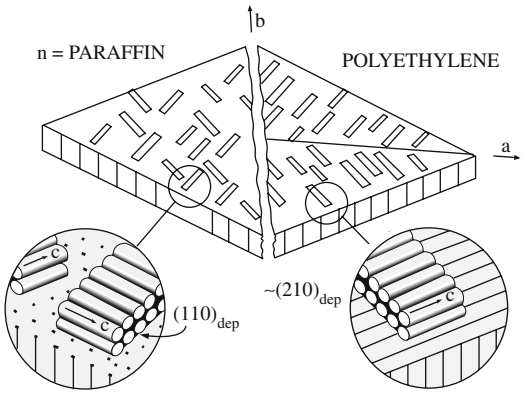

Fig. 7.6 Diagram of polymer decoration on a single crystal of paraffin (*left*) and a single crystal of polymer (*right*)

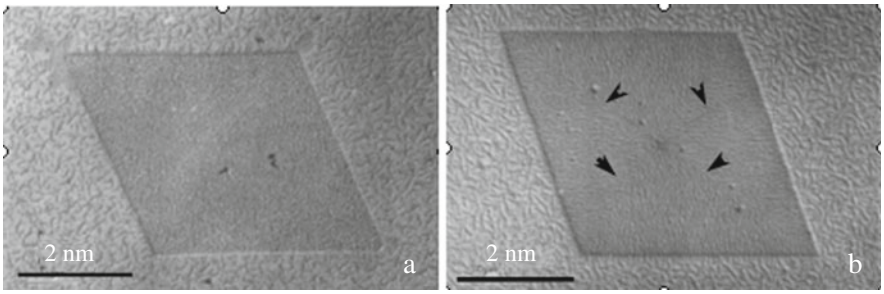

Fig. 7.7 Single crystals decorated using polyethylene (PE) vaporized under vacuum. (**a**) Orientation of PE lamellae on a single crystal of paraffin; (**b**) a single crystal of PE. On the PE single crystal, the orientation of the lamellae varies depending on the crystal-growth sectors, which is not the case for the paraffin single crystal. The *arrows* indicate the boundaries between growth sectors (*J.C. Wittmann, B.A, Lotz, ICS, Strasbourg*)

1.3 Variants

1.3.1 Bidirectional Shadowing

If the sample is very small (e.g., macromolecules), bidirectional shadowing is performed by successively orienting the sample in two directions at 90° (or 180°) from one another. If shadowing is performed at 180°, two evaporations of different thickness will be performed. The second layer must have approximately 10–20% the thickness of the first layer (Fig. 7.8). This process is used to make structural details appear within the normal shadowing angle. The metallic shadowing angle will be between 5° and 15° and will be even lower if the sample is flatter (Fig. 7.9).

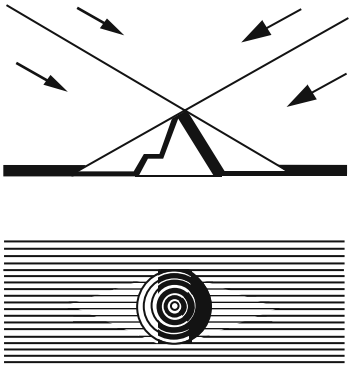

Fig. 7.8 Bidirectional shadowing at 180° with two different layer thicknesses

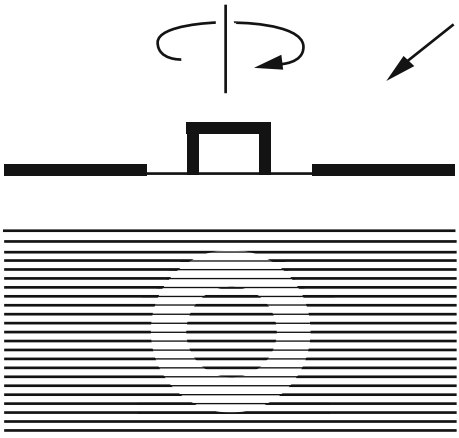

Fig. 7.9 Chromatin after bidirectional shadowing at 90° and dark-field observation (*E. Delain, Microscopie Moléculaire, Gustave Roussy-Villejuif)*

1.3.2 Rotary Shadowing

Rotary shadowing is done by turning the sample in its plane during metallic shadowing. The rotational speed is between 60 and 120 rev/min. The metallic shadowing

angle will be between 5° and 15° and will be even lower if the sample is flatter (Fig. 7.10). This technique is particularly useful for molecules in the form of filaments (DNA, RNA, and fibrillary proteins). This time, contrast is not obtained by shadowing, due to the lack of deposition in some zones, but rather by the differences in dispersion of metallic particles when they are deposited on the specimen. This technique is used to distinguish certain details of the specimen in all directions. It is particularly suited to investigate very small particles (Fig. 7.11).

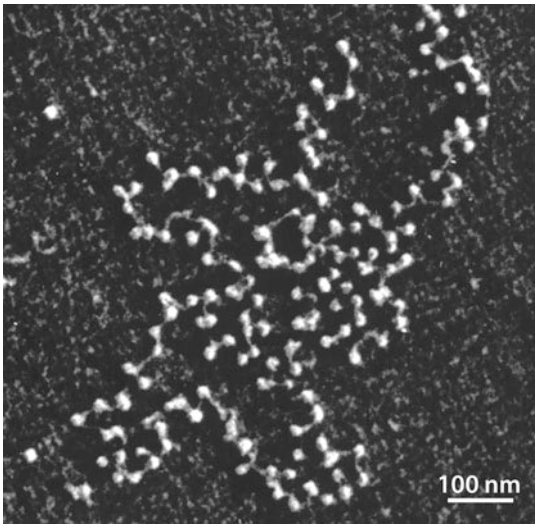

Fig. 7.10 Chromatin after bidirectional shadowing at 90° observed in dark-field imaging mode (*E. Delain, Microscopie Moléculaire, Gustave Roussy-Villejuif*)

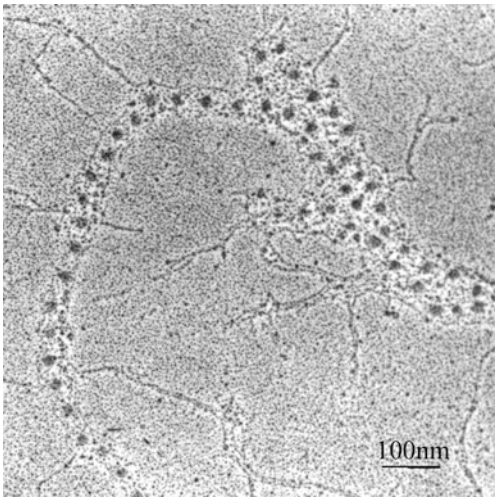

Fig. 7.11 Fibrillin in the shape of *stars*, grouped in *chains*. This is a component of cartilage. Rotary shadowing angle of 8° (*B. Burdin, CTμ, Université Claude Bernard-Lyon 1*)

1.4 Advantages

This technique is used to study fine particle or very small samples without contrast, such as macromolecules. It is used to see their spatial conformation after spreading. It is particularly effective for fibrillary or matrix structures and allows for 3D image reconstruction. It is also very useful for highlighting 2D crystals. It is used to measure particle thickness or the height of atomic steps of crystal growth (Fig. 7.12).

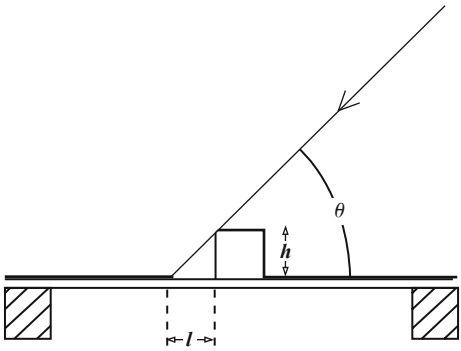

Fig. 7.12 Calculating the height or thickness of a particle: $h = l \cdot \mathrm{tg}(\theta)$

Since the angle, θ, of projection of the metal is known, we can measure the shadow length, l, of roughness, calculating its height, h: $h = l \cdot \mathrm{tg}\,\theta$.

1.5 Limitations

The main drawback is related to the risk of agglomeration of metallic particles, as resolution depends on their size and/or distribution. Furthermore, this technique is not well suited to investigating small, globular fine particles because it is difficult to distinguish them from the metallic particles.

Shadowing causes a shell to form around the object (Fig. 7.13). This shell is either lateral for directional shadowing or circular for rotary shadowing. It is bidirectional in the case of bidirectional shadowing. This shell increases particle size and

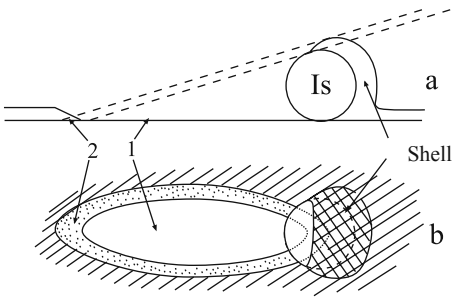

Fig. 7.13 Diagram of the formation of the envelope (shell) around the specimen: (**a**) object and (**b**) image after shadowing

must be considered when performing a dimensional investigation. The shadowing scope is also increased (Fig. 7.14).

Distribution of metallic grains can be irregular (Fig. 7.15) or the grains may also agglomerate, causing a loss of resolution (Fig. 7.16).

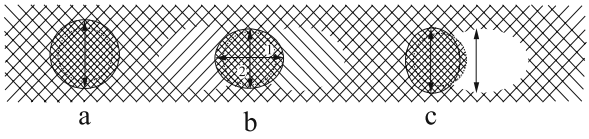

Fig. 7.14 Different shadowings: (**a**) rotary shadowing, (**b**) bidirectional shadowing, and (**c**) unidirectional shadowing

1.6 Artifacts

If the metallic deposit is too thick and leads to metallic particle aggregates that are a hindrance at high magnification (Fig. 7.16), the preparation background will present a visible grainy appearance, even at low magnification (Fig. 7.15). However, these defects can be avoided by using tungsten deposits (instead of the traditional platinum), which has less of a tendency to form aggregates. Thin deposits (a few nanometers) yield very low contrast shadowing in bright-field mode, but are highly visible in dark-field mode (Fig. 7.9).

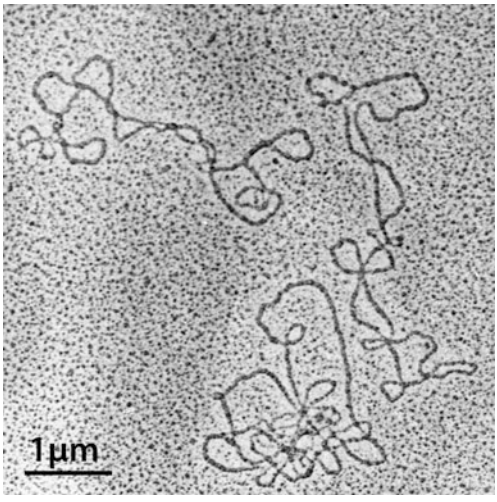

Fig. 7.15 Rotary shadowing of DNA with small irregularly distributed metallic particles aggregates (*E. Delain, Microscopie Moléculaire, Institut Gustave Roussy-Villejuif*)

1.7 Type of Analysis

Structure, crystal defects, crystallography.

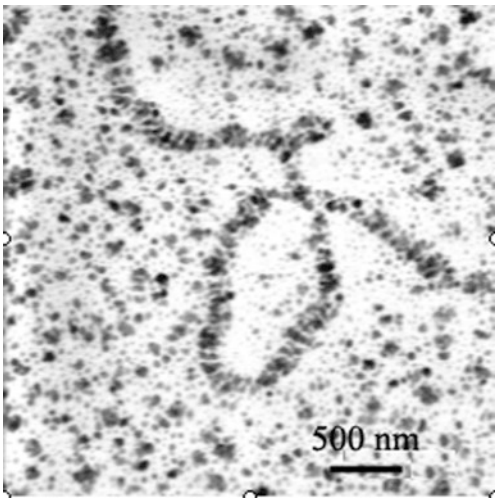

Fig. 7.16 Improper shadowing of DNA with small metallic particles aggregates hindering observation (*E. Delain, Microscopie Moléculaire, Institut Gustave Roussy-Villejuif*)

1.8 Risks

Risks related to the vacuum.

1.9 Conclusion

This is the reference technique for investigating fibrillary macromolecules (such as collagens, DNA, and RNA). It complements the fastest technique for increasing contrast using negative staining and the more complex frozen hydrated film technique. This is a widespread technique for investigating the topography of thin polymer films. For bulk materials, the replica technique is used, which is also based on shadowing but which assumes that the material is eliminated before observation.

2 Negative Staining

2.1 Principle

The technique consists of enhancing the contrast of a sample that is too thin or too transparent to the electrons to be viewed directly. This is generally the case for materials composed of light elements (C, O, H, N, etc.), as well as polymers and biological materials. Negative-staining contrast can only be applied to fine particle samples that are dispersed in liquid phase, preferably water. This technique consists of surrounding the sample with a chemical agent that is dense to the electrons, enabling the structures to be observed appear bright against a dark background. The

technique is performed by making a homogeneous deposit of a saline solution containing a heavy chemical element (tungsten, uranyl, molybdate, etc.). This is a very simple and rapid technique.

Negative-staining contrast does not produce any chemical reactions between the contrast agent and the sample, which is what differentiates it from positive-staining contrast. The only action is adsorption of the contrast agent on the support film and the sample surface. This adsorption is labile and can be canceled out by washing.

2.2 Operating Mode

2.2.1 Equipment and Supplies

No equipment is necessary for this technique. Standard laboratory glassware is used (Fig. 7.17a) (Petri dish, self-closing tweezers, precision micropipettes, etc.).

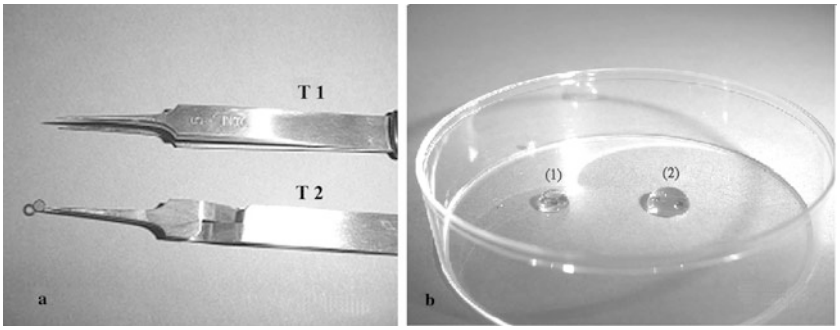

Fig. 7.17 The first two steps of negative staining are illustrated. (**a**) T1: a pair of straight tweezers; T2: a pair of self-closing tweezers holding a support grid for depositing the sample. (**b**) Petri dish with (1) a drop of suspension with a grid overturned on top and (2) the drop of stain

2.2.2 Procedure

Preparing the Sample

The fine particle material can be globular or fibrillary, but must be <5 μm in size and the material must first be dispersed in a solvent. The solution must be concentrated to ensure a sufficient amount of material, with a minimum of particle aggregation in one grid square (see Chapter 6, Section 1). Typically, this is a concentration of around 10^8–10^9 particles/ml.

Insofar as possible, dilution should be done in water. If necessary, this can be buffered and osmotically balanced for biological materials. For polymers, it is preferable to have a minimum of adjuvants, such as dispersants and wetting agents that can create artifacts in the preparation. Solutions that are too rich in salts should be avoided, as they will precipitate during deposition of the suspension on the grid. If the sample is suspended in fats, the fats must be eliminated because oils evaporate

with difficulty and are not stable under the electron beam. Lastly, the buffer in which the sample is suspended may not be miscible with the dye solution. The solution is dialyzed against a solution of 1–2% ammonium acetate or ammonium carbonate.

Dialysis is a technique for purifying solutions between two liquid media separated by a semi-permeable membrane that is often presented in the form of dialysis tubing. The membrane has pores on the order of a micron in diameter. Through the effect of osmosis and molecular agitation, the small molecules cross the membrane while the large molecules (macromolecules) are kept in the dialysis tubing.

For very fragile biological samples, it may be necessary to perform prior fixation, e.g., using aldehydes. Paraformaldehyde is used for viruses because it is considered a virulence deactivator.

Immunolabeling can also be performed before depositing the drop of suspension.

Type of Support Grid

A tight-mesh support grid (400–600 mesh) is used, which must be covered with a continuous carbon or polymer support film (see Chapter 2, Section 13). The film's surface must be wettable and electrically charged on the surface. Theoretically there are no problems with polymer films. Formvar is preferred over collodion for better resistance to the weight of particles and greater stability under the electron beam. Freshly made carbon films are hydrophobic. Aging over a few days or a few weeks will enable the samples to become electrically charged. This operation can be performed quickly using a "glow discharge" by creating an ionization current in a partial vacuum. Depending on the residual gas in the chamber where the discharge is performed, positive or negative ions could be deposited on the film surface. This allows the operator to choose the type of ions required, with an opposite charge to those of the sample to be stained. For example, for negatively charged nucleic acids such as DNA, ionization in the presence of pentylamine (Dubochet device) results in a positively charged carbon grid and thus allows the DNA molecules to spread out.

Methods

Depositing the sample on the support grid can be done in three ways.

Method 1: The grid is turned over onto a drop of the preparation placed in the bottom of a Petri dish (or on a piece of plastic film) and is left in contact with it for 1–2 min (Fig. 7.17b). The smaller the size of the particles, the longer the time needed to recover them. In this situation, the heavy particles quickly fall to the bottom of the drop and only the lighter ones adhere to the film on the grid. A heat source placed nearby promotes Brownian movement within the drop, which promotes adhesion of a greater number of particles. For a better result, it is important that the grid has a large contact area with the drop, which occurs if the drop is spread out broadly on the surface of the Petri dish and the grid is at an angle around 45° with regard to the drop's meniscus.

After the required time has elapsed, the excess liquid solution is blotted briefly using a filter paper corner and the grid is immediately turned over onto a drop of

contrast agent for 20–30 s. Then, with a filter paper corner, the excess water is soaked up onto the grid while keeping it in the tips of the tweezers (Fig. 7.18).

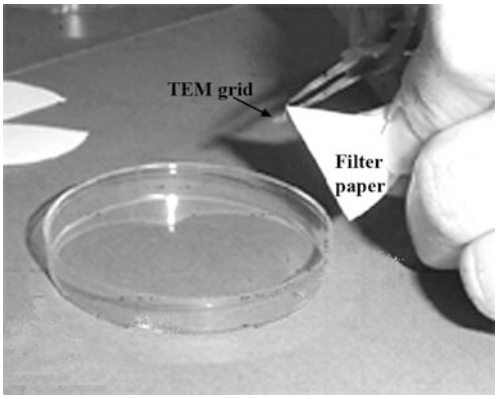

Fig. 7.18 Illustration of the third step: Absorption of contrast agent by the filter paper

Figure 7.19 illustrates the entire procedure.

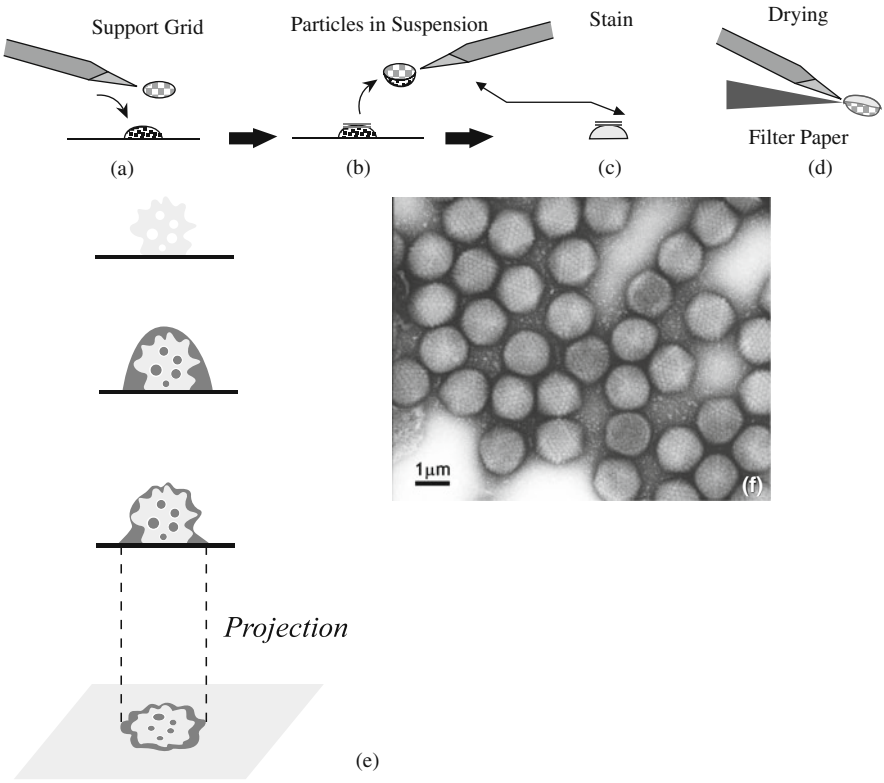

Fig. 7.19 (**a**–**d**) Diagram of the procedure. (**e**) Diagram of 2D projection of particles after staining. (**f**) TEM bright-field image of adenovirus particles (*E. Delain, CNRS-UMR8126, IGR Villejuif)*

Method 2: The suspension drop can be placed directly on the support grid held in a pair of self-closing microcapillary tweezers (Fig. 7.17a): The self-closing microcapillary tweezers help to prevent the drop from rising up between the jaws of the tweezers. Thus, a known quantity (e.g., 5 μl) of the solution can be deposited and left until the solvent completely evaporates. All of the particles in the initial drop of suspension will end up on the grid. A statistical study of the deposit enables the determination of the quantity of particles contained in a known volume and therefore the determination of the concentration of particles in the solution. In this configuration, the evaporation rate of the drop can be increased by placing the self-closing tweezers near a heat source (e.g., a lamp).

When the drop of liquid is totally evaporated, but before it dries, the grid is then turned over onto the drop of contrast solution (as in the previous method).

The contrasting time is very short in these first two methods in order to prevent any possibility of a chemical reaction between the sample and the contrast agent, i.e., any possibility of positive contrast. It is typically around 20–30 s. Extending the time will not improve the quantity deposited and therefore contrast quality. In order to facilitate the fast recovery of the grid, it is constantly held between the jaws of a pair of tweezers during the contrasting procedure. Excess contrast solution is blotted with a filter paper, as in the previous method. After 10 min of air-drying, the sample is ready for observation.

Method 3: For particularly brittle samples, the contrast agent can be mixed directly with a small quantity of suspension before placing this mixture on the support film of an observation grid. Reaction control time is more difficult to achieve. This technique is used especially with PTA (phosphotungstic acid).

Contrast Agent

This is a heavy-metal salt (W, Mo, U) with a concentration of 0.5–2% in water, adjusted to a pH corresponding to the sample dilution medium and the required electrical charges (see "Methodology", Chapter 4, Section 3 of this chapter).

Phosphotungstic acid (PTA) is used at 1–2% dissolved in distilled water. The concentration can be as low as 0.5%. The solution's pH is adjusted to 7 with KOH at 0.1 M (rather than NaOH). In fact, the contrast agent is potassium (or sodium) phosphotungstate ($24WO_3 \cdot 2H_3PO_4 \cdot 48H_2O$) and was introduced by Hall in 1955. It generates anions.

Sodium silicotungstate ($Na_4(Si[W_3O_{10}]_4) \cdot 20H_2O$) is used at 1% in distilled water. Its concentration can vary between 1 and 4% with a pH between 5 and 8, usually 6.8. It was introduced by Valentine et al. in 1966 and provides good resolution around 1 nm.

Ammonium molybdate ($(NH4)_6MO_7O_{24}$) is used at 4% dissolved in distilled water. The concentration can vary from 0.5 to 5%; the pH ranges from 5 to 8 and is adjusted with NH_4OH to 0.1 M. It was introduced by Munn in 1968. An example with this contrast agent is shown in Fig. 7.20.

Methylamine tungstate ($CH_3NH_2WO_3$), introduced by Fabergé and Oliver in 1974, is difficult to prepare.

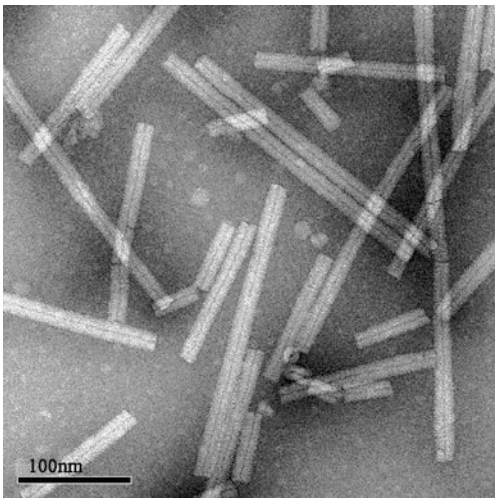

Fig. 7.20 Bright-field TEM of the tobacco mosaic virus (TMV) in 3% ammonium molybdate and 1.5% uranyl acetate (*P.Y. Sizaret, Université Tours)*

Uranyl acetate ($C_4H_6O_6U$) is used in much lower concentrations, from 0.1 to 2% for positive staining. Furthermore, the pH is between 4 and 5.5. Uranyl acetate and its derivatives were introduced by Van Bruggen et al. in 1960. They generate cations. It is also a fixative and sometimes reacts with structures, creating positive staining. Examples using this contrast agent are shown in Figs. 7.21 and 7.22.

Aurothioglucose is recommended for RNA. It was introduced by Kühlbrandt in 1982.

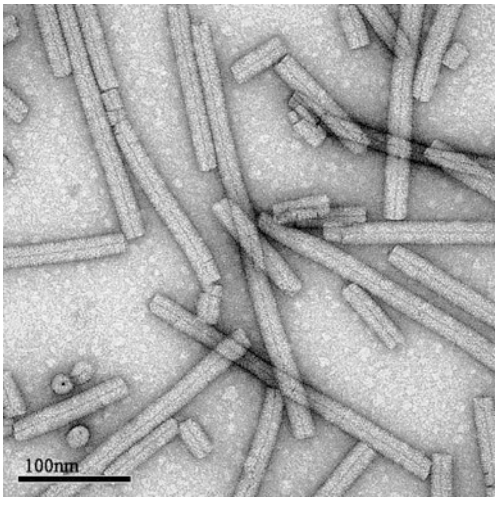

Fig. 7.21 Bright-field TEM of the TMV in 1.5% uranyl acetate (*P.Y. Sizaret, Université Tours*)

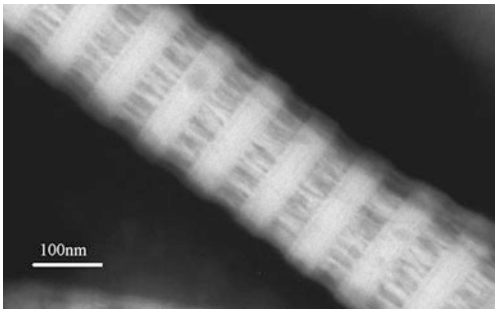

Fig. 7.22 Collagen fiber with negative staining by uranyl acetate viewed in TEM bright-field mode (*A. Rivoire, EZUS UCB-Lyon 1*)

It is always recommended to make the contrast solution extemporaneously, but experience shows that it can be kept in the refrigerator without problems, in particular for PTA. However, it is necessary to check pH before use. Examples with PTA contrast agent are shown in Figs. 7.23 and 7.24.

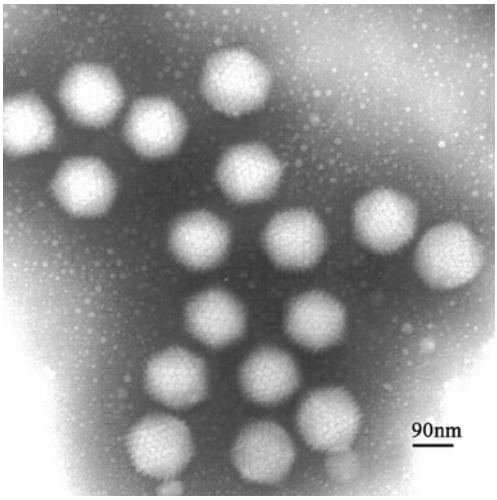

Fig. 7.23 Adenovirus, negative staining with PTA, TEM bright-field mode (*E. Delain, CNRS-UMR8126-IGR, Villejuif*)

2.2.3 Observations

The obtained grids can be observed directly after a short drying time. The specimen is surrounded by heavy metallic atoms that act as a barrier to the electrons. Since electrons pass through the low electronic density of the sample, but not through the metallic background, the result is a bright image of the sample on a dark background.

The final image is influenced by the thickness of the contrast agent, which depends on the wettability of the support film, the quantity of particles of the sample adsorbed, the quantity of water remaining in the contrast agent, and the properties of the contrast agent.

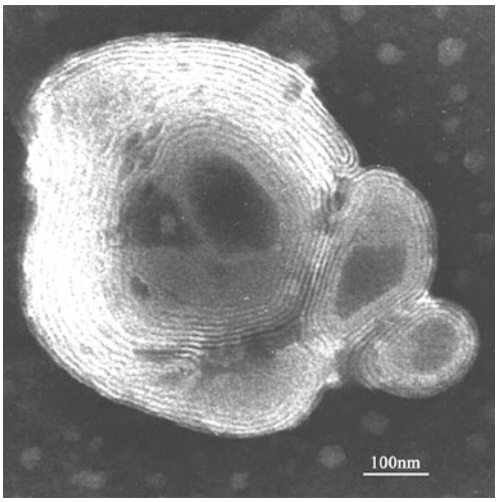

Fig. 7.24 Highlighting liposome flakes, negative staining with 1% PTA, pH 7, TEM bright-field mode *(J. Boumendil, UCB Lyon 1)*

The film quality of the deposit depends on the reciprocal polarity of the sample and the support film.

These deposits are relatively unstable under the electron beam (see Fig. 7.25), which can cause irradiation damage. During observation in the microscope, it is necessary to gradually illuminate the sample in order to prevent water evaporation due to the thermal effect produced under the beam and prevent excessively high electron doses. The uranyl deposit is more unstable than phosphotungstic acid (PTA) under the electron beam.

For viral particles or particular specimen it is difficult to choose the good contrast agent and often a mixture of two agents is a good solution (as shown in Fig. 20).

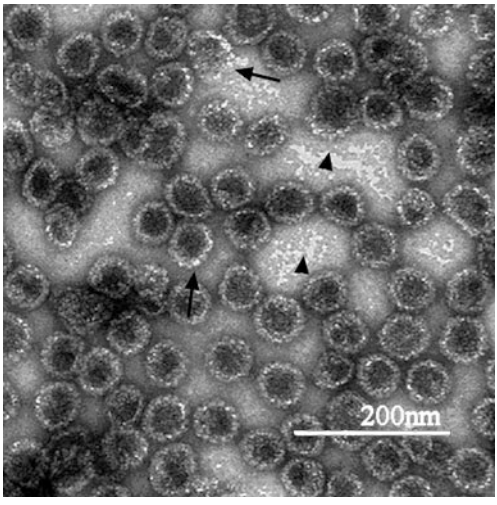

Fig. 7.25 TEM bright-field image showing viral pseudo-particles with irradiation damaged (*arrows*) formed under the electron beam (*P.Y. Sizaret, Université Tours*)

2.3 Variants

There are no variants.

2.4 Advantages

This technique is very rapid. It allows a good approach to the size and shape of single particles deposited, as well as certain surface details analysis. It is very useful in virology and nanoparticles engineering. It is used to differentiate the original and coalescent shapes or agglomerated shapes, which is not possible with light diffusion. Additionally, the use of the technique of depositing a known quantity (the second method) allows us to determine the concentration of particles in the initial solution. The measurement obtained is comparable, even better than that obtained by light diffusion, which does not take into account aggregates and is very close to titration measurements practiced in virology.

2.5 Limitations

This technique can be defective if there is no compatibility between the sample, the support, and the contrast agent.

Most contrast agents used in this technique restrict observation of details to around 1–2 nm. The presence of components other than water in the suspension can sometimes interfere with observation.

2.6 Artifacts

Many artifacts are possible and must be detected.

The thickness of the contrast agent deposited on the grid constitutes an important factor in observation and precision. This depends on the wettability of the film, the way the excess contrast agent is blotted, and the quantity of sample particles deposited on the film. Too much material, e.g., covering all of the film, will hinder contrast agent deposition. There will be no improvement in contrast in this case. It is usually preferable to have too high a dilution rather than too strong of a concentration, as is shown in Fig. 7.26.

Some parameters that are hard to control can interfere with the size of the material to be investigated:

– The type of contrast agent
– The method of deposition
– Osmotic variations between the two solutions can cause size and shape changes
– Slight desiccation of the material deposited on the grid film

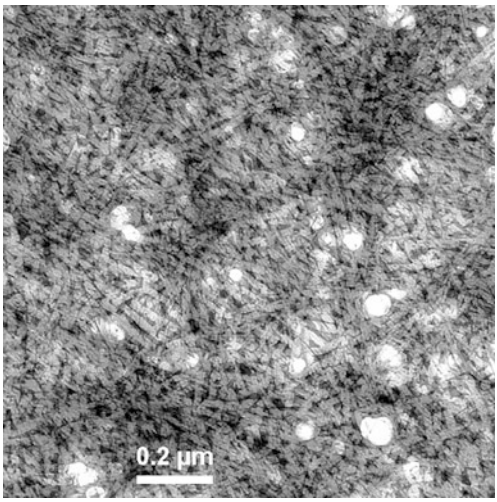

Fig. 7.26 TEM bright-field image of the TMV in 5% uranyl acetate, showing TMV particles concentration that is too high, which does not enable the individual viruses to be distinguished on the contrast agent film (*P.Y. Sizaret, Université Tours*)

Some contrast agents can crystallize under certain concentration or pH conditions. These crystals deposited on the grid will hinder observation.

Negative contrast is very sensitive to irradiation by the electron beam. Attention must be paid so that the preparation is thoroughly dry before being placed in the microscope, and a low-beam intensity must be used. Otherwise, the contrast agent sublimates and areas of the preparation will appear without contrast (Fig. 7.25).

2.7 Type of Analysis

Topography, structure, crystal defect, crystallography, properties

2.8 Risks

Uranium salts are radioactive and chemically toxic. However, there is no risk at the concentrations used.

2.9 Conclusion

A contrast agent can prove very useful for one type of material and disappointing for another. It is necessary to try several different contrast agents, several pH values, and different concentrations.

This is an excellent, routine technique whose resolution limit is from 1 to 2 nm. Better resolution will be obtained by the shadowing techniques or direct observation in frozen hydrated film solution. Widely used by biologists, this technique is finding

new applications in viewing nanoparticles and other liposomes, making it possible to quickly check their shape and size depending on the manufacturing process.

3 Positive Staining

3.1 Principle

This technique is used to enhance contrast in a sample. It is based on the chemical reaction between a heavy metal salt (uranium or lead) and the constituents of the material. This technique is applied to materials composed of light elements, fine particles, or bulk samples thinned using the ultramicrotomy technique. It is applied especially to polymers and biological materials.

3.2 Operating Mode

3.2.1 Equipment and Supplies

The technique can be performed without any special equipment. However, there are commercially available devices for processing several preparations at once, with greater control and reproducibility than when performing the procedure manually.

3.2.2 Procedure

Bulk Materials

Staining Ultrathin Sections of Polymer Samples

In this case, highly volatile oxidants (ruthenium oxide and osmium tetroxide) are mainly used, which act on the double bonds of the polymer. The preparation is deposited on a nickel support grid (rather than copper, which is easily oxidized). These reagents are used in aqueous solution placed in a confined enclosure (Petri dish or closed pillbox). The grid is placed near a drop of liquid without touching it; only the vapors emitted by the oxidant will react with the sample. *Caution:* These vapors are highly oxidizing and therefore very toxic. It is essential to work under a fume hood, wearing protective goggles. The preparation is left in the presence of these vapors from 1 to 24 h, depending on the sample type. After removing the support grid from the confined enclosure and letting the residual vapors escape under the hood, the sample is ready for observation.

For certain less reactive polymers, it may be necessary to perform this operation at 310 K or even 333 K. In this case, a special enclosure is required in order to prevent any risk of these oxidizing vapors dissipating.

Table 7.1 provides a few examples of staining solutions depending on the chemical nature of the polymer.

Table 7.1 Different staining solutions as a function of the chemical nature of the polymer

Polymer	Stainig solution
Hydrocarbons, alcohols, ethers, unsaturated amines	Osmium tetroxide
Ethers, alcohols, aromatics, gums, biphenol, styrene	Ruthenium tetroxide
Acids or esters	Hydrazine, then osmium or uranyl acetate
Unsaturated gums	Ebonite
Saturated hydrocarbons (PE and PP)	Chlorosulfonic acid/uranyl acetate
Amides, esters, and PP	Phosphotungstic acid
Esters, aromatic polyamides	Silver sulfide

Staining Ultrathin Sections of Biological Materials Embedded in Epoxy Resin

Double staining is usually performed in this case and involves a uranium salt followed by a lead salt.

Uranyl acetate is a toxic substance that is often faintly radioactive (emitting γ-radiation). It must be handled with caution, especially the initial powder, which is fine and volatile.

(i) *Uranyl acetate* is dissolved in a concentration nearing saturation between 0.5 and 9%, depending on the solvent used (water, ethanol, and methanol). Higher concentrations result in more contrast. Saturation is on the order of 2% in water, 7% in ethanol, and close to 9% in methanol. Methanol is a highly volatile alcohol. The solution can be prepared in advance, but the storage flask must be well stoppered and sheltered from light. Uranyl acetate is unstable in the light. At the time of use, the needed quantity of solution is filtered on filter paper (Fig. 7.27).

The grid is placed so that it is touching the solution for 5 min to several hours either at room temperature or heated (up to 333 K) in a closed chamber. This is done in order to prevent any evaporation, which would cause the formation of needles of uranyl salt on the preparation by precipitation. If the staining time exceeds 5 min, then staining must also be carried out sheltered from the light.

Once the staining time has run out, the grid is immediately rinsed with the solvent corresponding to the initial solution, followed by water. Several rinses are necessary to eliminate any residual traces of stain from the material. Rinsing is performed either by breaking the liquid's meniscus several times with the grid, in three successive baths, or with a water jet directed to the grid at an angle of approximately 30–45°. The grid is then dried for a few hours before beginning the second staining.

Comments: Sections made on materials embedded in epoxy resins are difficult to contrast because these resins are not very permeable. For epoxy resins, it is recommended to use uranyl/alcohol solutions near saturation and sometimes to perform staining at 333 K. For very dense materials, it is preferable to use 3–5% solutions in ethanol.

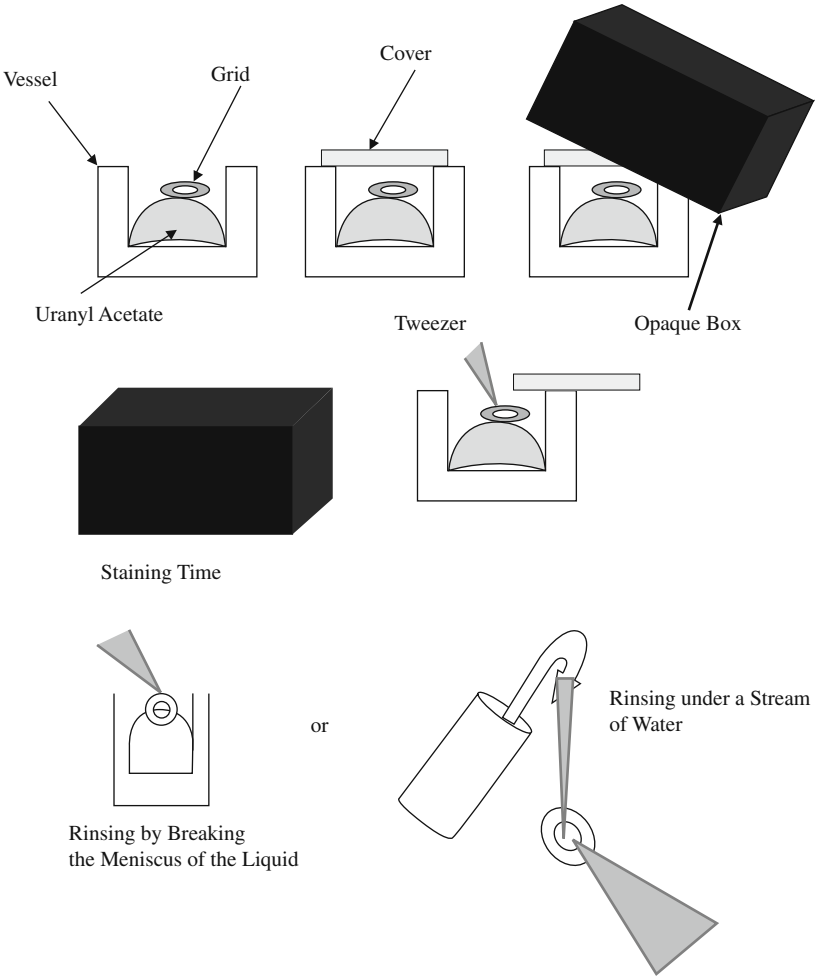

Fig. 7.27 Different steps of staining with uranyl acetate

A solution of uranyl in water at a concentration of 1–2% is used for slices made of materials embedded in acrylic resins, which are usually polar and permeable (K4M and K11M). If the tissue has not undergone osmotic fixation and has been frozen, staining is performed with a solution of 0.2–0.5% uranyl acetate in water. Keep in mind that in this case, contrast is inverted; membranes normally contrasted by osmium are transparent to the electrons and the uranyl colorant is fixed on cellular matrix.

(ii) *Lead citrate staining:* This second reagent is a salt obtained by a reaction between lead nitrate and sodium citrate in aqueous solution at a pH of around 12. Commercially available lead citrate is not satisfactory. Staining is performed in a

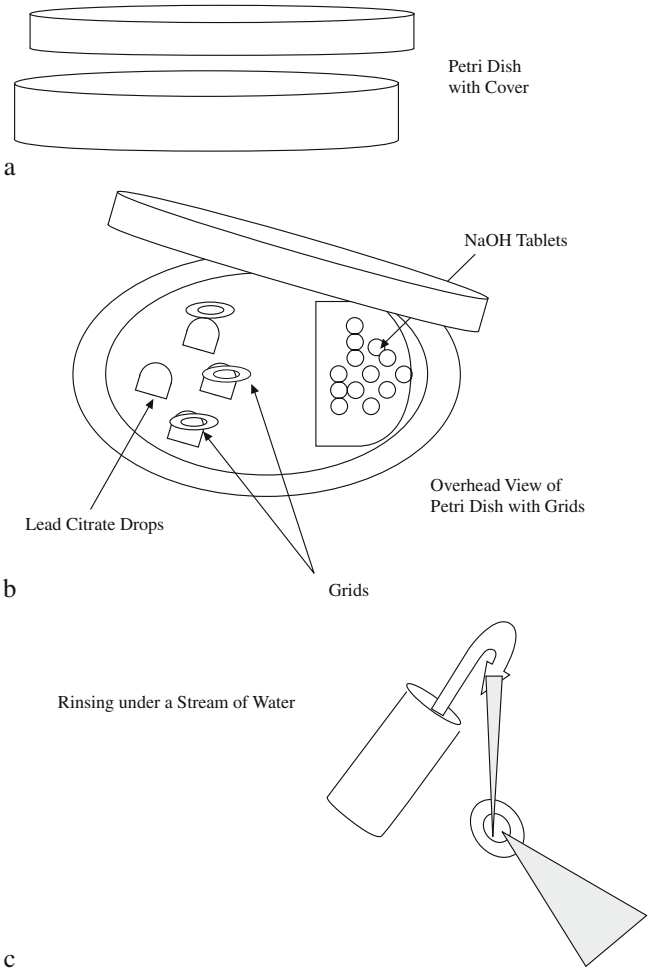

Fig. 7.28 Different steps of contrasting with lead citrate

sealed enclosure (plastic Petri dish) containing soda (NaOH) tablets in order to produce a dry, alkaline atmosphere, lacking in CO_2 (see Fig. 7.28).

Grids are placed specimen side down onto drops of staining solution for a short time, from 1 to 5 min. It is important to avoid breathing over the grids in order to prevent the addition of CO_2 in the atmosphere, which results in lead precipitates. The grid is rinsed immediately with distilled water and then allowed to dry before observation.

Double staining can be performed using commercially available devices. The simplest ones contain only a small enclosure like a grid box, which is used to stain tens of grids at once. The most sophisticated models are truly automatic: These are contrasting instruments with colorant and rinsing liquid distribution and heating

systems. They can perform double staining without the operator being involved once the grids are placed in the device. Contrast solutions are available from the device's manufacturer, thereby ensuring good reproducibility.

Fine Particles: Staining Particles in Solution

In this case, contrasting is performed immediately after the suspension is deposited on the film-covered support grid (see Chapter 6, Section 1) and before the preparation has completely dried.

One technique consists of turning the grid over onto a drop of uranyl acetate in aqueous solution (1–2%) for 1–5 min before it is rinsed, as in the previous operation. After staining with uranyl acetate, contrasting with lead citrate can be performed if necessary. When this contrast agent is used, staining is always carried out on a dry grid. Usually, a single contrast agent (uranyl and lead) is enough.

3.3 Variants

3.3.1 Variant for Spread-Out Molecules

If the support grid with the dispersed sample is loaded with NH^+ particles on the surface, according to the Dubochet technique (see Chapter 6 , Section 1), staining can be performed on the support grid by simply placing a drop of uranyl acetate with the grid tilted so that the drop slides down quickly. The grid is immediately dried on filter paper to remove excess contrast agent. It is now ready for observation (see Fig. 7.29).

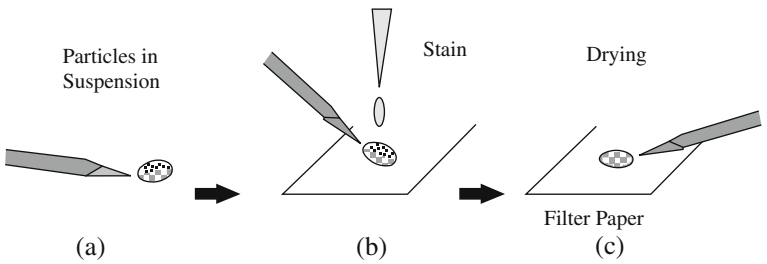

Fig. 7.29 (**a**–**c**) Positive-staining steps using uranyl acetate on fine particle samples

It is not necessary to rinse the grid in this type of preparation because uranyl is positively charged and cannot stick to the surface of the film; therefore negative contrast cannot be obtained. Positive contrast is obtained when uranyl fixes to the negative sites of the DNA. The grid is then ready for observation (Fig. 7.30).

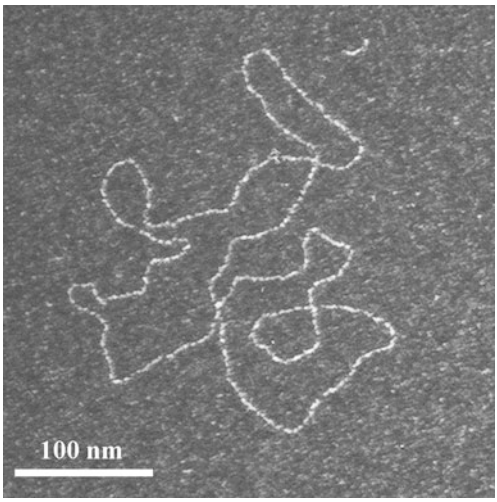

Fig. 7.30 Zero-loss TEM image in annular dark-field mode of relaxed circular DNA of plasmid pBR 322, 4361 bp (*E. Le Cam, CNRS-UMR8126, IGR Villejuif*)

3.4 Advantages

This is a rapid technique for enhancing contrast in electron-transparent samples. Fixation of heavy metals occurs at specific reactive sites, which results in marking only specific areas, depending on their chemical structure, so they can be differentiated.

For polymers, reaction with oxidants (Ru, Os) occurs at the double bonds (Fig. 7.31). It helps to differentiate two polymers when one has saturated bonds and the other does not.

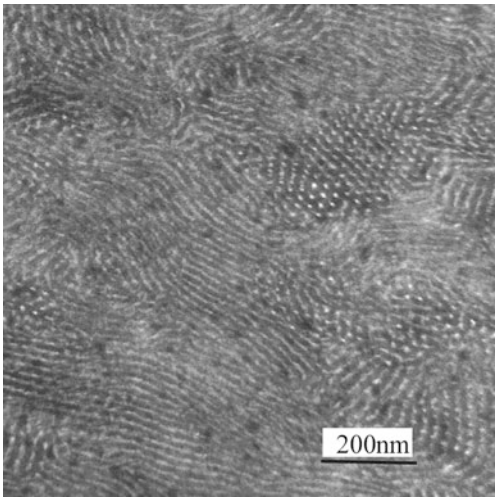

Fig. 7.31 Mixture of two polymers contrasted with ruthenium tetroxide, which has darkened one of the two phases (*A. Rivoire, Ezus, UCB Lyon 1*)

With DNA, uranyl fixes to the sugar-phosphate chains (Fig. 7.30). This mechanism is poorly understood for biological materials that have already undergone double fixation. It appears that these contrast metals (uranyl or lead) attach to sites already containing osmium radicals. It can clearly be seen how useful this technique is by comparing an image made on a section without coloration with one that has undergone double contrasting (Figs. 7.32 and 7.33).

This is a key technique for facilitating observation of organic materials (non-calcified). It is performed after fixation, embedding, and cutting into ultrathin slices.

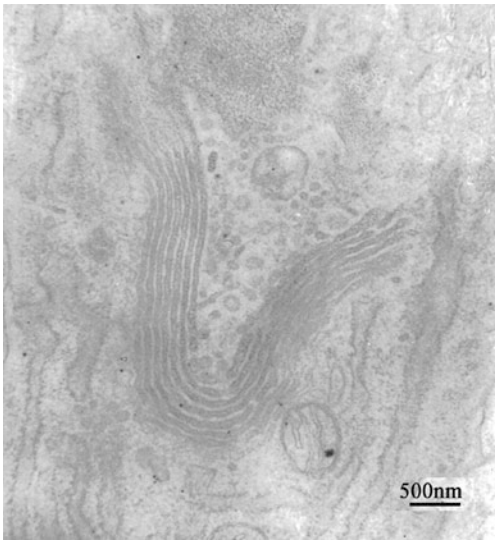

Fig. 7.32 Golgi apparatus and mitochondria without positive contrast (*J. Boumendil, Université Claude Bernard-Lyon 1*)

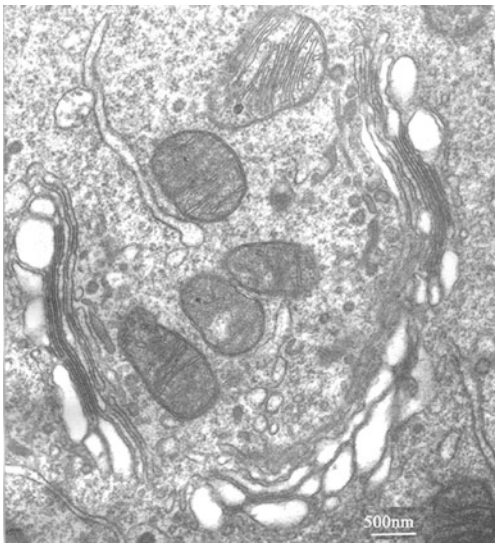

Fig. 7.33 Golgi apparatus and mitochondria after double-positive contrast with uranyl and lead (*J. Boumendil, Université Claude Bernard-Lyon 1*)

3.5 Limitations

This technique can be used only if the sample has reactive sites, e.g., double bonds in polymers. The specificity of these reactive sites is poorly understood chemically and the result is therefore not always predictable. The chemical composition of the sample is modified by the addition of heavy chemical elements that can hinder X-ray analysis. In fact, osmium, uranium, or lead emits X-rays (L and M lines) that are situated near the chemical elements in which we are interested, e.g., calcium, sodium, phosphorous.

3.6 Artifacts

As positive staining is the final step in preparing bulk samples, it amplifies all of the defect:.

- It can amplify the volume of osmium precipitates, which look like dense particles distributed randomly on the surface of the sample. These are referred to as "pepper" grains. This artifact is due to excessive deposits (adsorbed and not fixed to the structures) during osmotic fixation. Lead′s special affinity for this type of deposit increases the size of these particles that are more unstable under the electron beam. In order to prevent this phenomenon, the sample needs to be placed in distilled water after osmotic fixation and before dehydration.
- It amplifies oil or dust residues coming from the water used to pick up the sections after ultramicrotomy. To remedy this, the water contained in the trough needs to be changed very frequently, each time that particles or greasy patches are seen under the stereoscopic microscope.

Contrast agents can also react weakly with embedding resins, which highlights the light graining they may have.

The two successive contrasts can generate electron-dense precipitates. Uranyl precipitates are needle shaped (Fig. 7.34), while lead precipitates are spherical and present a cross shape, highlighting an orientation under the electron beam (Fig. 7.35).

Uranyl precipitates are due to the contrast solution drying on the preparation after rinsing. This occurs more often with contrast agents in alcohol solution (particularly with highly volatile methanol) and if contrasting is performed using high temperature (310–333 K) that results in evaporation. Proper handling, with a hermetically sealed contrast vessel and immediate immersion of the preparation in the rinsing solution, can help eliminate this artifact.

The solution for lead citrate contrasting is clear if the pH is around 10. Below that, the solution is unstable and forms precipitates. Lead precipitates are prevented by working in an atmosphere completely free of humidity and CO_2 traces so as to not acidify the contrasting solution. To do this, work is performed in an enclosure

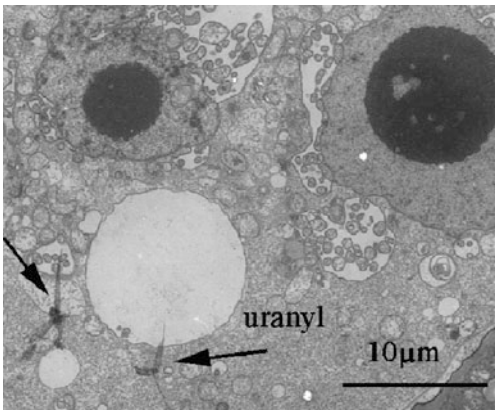

Fig. 7.34 Needle-shaped uranyl precipitates. Embryonic cells of a plantule (*J. Boumendil, Université Claude Bernard-Lyon 1*)

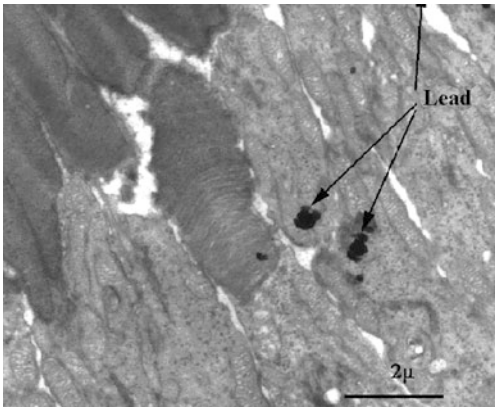

Fig. 7.35 Spherical lead precipitates (*J. Boumendil, CMEABG, Université Claude Bernard-Lyon 1*)

containing tablets of soda and the sample is sheltered from the operator's exhalation. The first rinsing bath is performed with slightly alkaline water (containing soda in solution at M/20).

3.7 Type of Analysis

Topography, structure.

3.8 Risks

There are toxic risks due to inhalation or ingestion of solvents (methanol, etc.) and heavy metals (especially lead). There are risks of respiratory irritation caused by

oxidant vapors. There are carcinogenic risks due to radioactive uranium radiation. This method requires working under a fume hood.

3.9 Conclusion

This is a routine technique for biological materials as well as polymers. For multiphase polymers, it is used to differentiate saturated and unsaturated polymers.

The same heavy atoms (osmium and uranium) can be used for chemical fixation, negative staining contrast, and positive staining contrast of biological materials. The difference essentially results from the reaction time: several hours for fixation, a few minutes to 1 h for positive staining (followed by a rinse), and a few tens of seconds for negative staining (without rinsing).

4 Immunolabeling

4.1 Principle

This technique consists of making a specific label in order to locate a molecule with a particular function inside the biological structure, enabling a structural analysis related to functional properties. The technique is based on the recognition of an antigen by an antibody. Immunological labeling reproduces the natural reaction every living organism has when confronted with a foreign molecule. This reaction consists of producing a specific immunoglobulin, called an antibody, which blocks the foreign molecule, called an antigen.

In immunolabeling, the molecule to be recognized is usually a protein or an amino acid, but it can also be another kind of molecule as long as a specific antibody can be produced. For transmission electron microscopy, this antigen–antibody combination is revealed by gold particles bound to the antibody. The electron-opaque gold particles are particularly easy to spot during TEM observation. Theoretically, their presence indicates the existence of the organic molecule of interest, but it is necessary to check that there are no gold particles bound non-specifically to the structures constituting what is referred to as labeling background noise. Sometimes quantification techniques are required in order to statistically bring out the labeling.

4.2 Operating Mode

4.2.1 Equipment and Supplies

No equipment is necessary for the technique itself, but the reagents do need to be made. However, if immunolabeling is performed on a thin slice, it may be necessary to make slices from a few hundred nanometers to a few hundred microns thick. A

cryo-microtome, a vibratome, an ultramicrotome, or a cryo-ultramicrotome can be used to do this.

4.2.2 Procedure

The overall principle of the reaction is shown in Fig. 7.36.

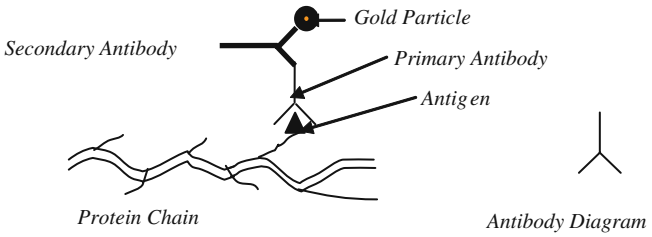

Fig. 7.36 The antigen–antibody reaction

For example, the molecule to be labeled is a specific chemical ending of a protein, which immunologists call an epitope. The primary antibody, e.g., produced in mice, will recognize this epitope (and only this epitope) and fix onto it. In turn, the second antibody directed against the mouse antibodies will fix onto the primary antibody from the mouse cells. Because the gold particle is fixed to the second antibody, transmission electron microscopy can be used to pinpoint the position of the epitope being looked for and locate it in the cell being investigated. Therefore, a complex has been created that is firmly attached to the antigen protein + primary antibody + secondary antibody + gold particle, as illustrated in Fig. 7.36.

Antibodies

Two antibodies must be used to perform the technique: a primary antibody produced to recognize the molecule to be detected, i.e., the antigen, and a secondary antibody that will be coupled to the gold particle.

The first antibody can be either a polyclonal or monoclonal antibody, depending on the production method. Monoclonal antibodies from a single cell are usually purchased from specialized catalogues, if available. This is more specific and is directed against a single epitope. Polyclonal antibodies are not used unless the monoclonal antibody required is not available on the market. In this case, an animal such as a rabbit is immunized against the molecule to be investigated. Its serum is collected, purified, and the antibody is concentrated. Its specificity is tested, but there is always a risk of it being reactive with several neighboring epitopes. In both cases, the primary antibody is always produced with the help of cells from an animal species: rabbits, mice, and usually rats. Obviously, a different animal species must be used than that of the molecule to be labeled, and the species must present the least risk of causing parasitic labeling.

The second antibody is an antibody directed against the antigens of the animal used to produce the primary antibody: this secondary antibody will be anti-rabbit, anti-mouse, or anti-rat. Usually it is made in goats. This second antibody is sold after being coupled with gold particles. The gold particles chosen have diameters that range from 1 to 20 nm.

In the labeling complex, gold particles have a relatively larger steric hindrance than the antibodies, which are very small molecules. Because of this, the size of the gold particles has an influence on labeling intensity. If we suppose that the epitope is found several times on the molecule at short distances, the steric hindrance of the gold particle can prevent another antibody fixation in a neighboring area. Therefore, the gold particle diameter has an important influence on the result. The larger the gold particle, the more the risk of labeling being faint. But very small particles (1–2 nm) are often hard to distinguish on tissue sections, so the most common particle size is 5–10 nm. Particles as large as 20 nm are used, especially for labeling when scanning electron microscopy is needed. The advantage of different particle sizes is that it enables double, and even multiple, labeling, so that two or more different epitopes can be located on the same sample, with each one labeled using a different particle size.

Therefore, antibody selection is an essential step in the technique.

The Sample

In order for the reaction to occur, it is necessary for the epitope to be accessible, i.e., the protein's spatial conformation must not be changed by the preparation prior to labeling.

The molecules to be highlighted, i.e., the antigens, are very sensitive to preparation techniques that can denature them, change their spatial conformation, and thus mask either the epitopes or eliminate them by extraction. The coagulating action of fixatives or dehydration fluids, like high temperatures required for polymerizing resins, is particularly harmful.

Therefore, there is always a loss of antigenic sites on tissue prepared for transmission electron microscopy. The significance of antigen loss resulting from the chemical preparation technique will depend on the antigen's position within the structure.

The diagram in Fig. 7.37 shows six possible antigen locations.

The best protected antigens are those trapped in the cell. They are located in a second compartment, such as the nucleus, mitochondria, or the vacuoles, and they are usually sufficiently preserved to have significant labeling. Free antigens in the cytoplasm are more sensitive to this technique. The same is true for antigens that are attached to the plasmic membranes; however, their bond with well-stabilized structures, such as membranes, provides a certain degree of protection (if not against denaturing, then at least against extraction). On the other hand, antigens that are in the *milieu intérieur,* i.e., the extracellular connective web, are much more easily extracted by dehydration.

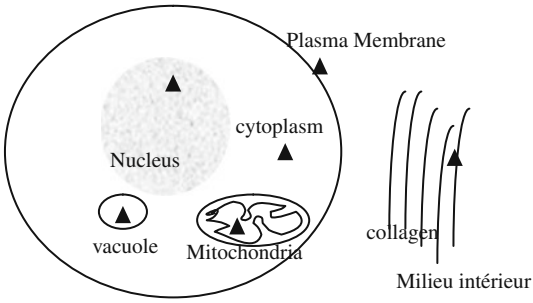

Fig. 7.37 Location of different antigens within a tissue

In difficult cases, and in all cases when the presence of an antigen is naturally low, physical preparations will be used in order to avoid one or more of the three steps of the chemical techniques: chemical fixation, dehydration, and hot embedding.

Antibody penetration of the antigenic site is another technical problem to be solved. The more protected the antigens are in the cell, the more difficult it is to access them. The membranes must be permeabilized or thin sections must be made in order to reach the center of the cells to bring the structures to the surface.

Method

This technique is performed on cells in suspension, histological tissue slices, ultrathin sections, or crushed frozen tissue. The resulting sample will be handled according to the following procedure, which includes several steps; all of the steps are usually carried out with regard to the pH and osmolarity of the sample through the use of a phosphate buffer.

(a) *Preliminary steps:* These steps may vary depending on the type of sample:

- *Neutralize the aldehyde groups:* If there has been an initial chemical fixation, leave for 10 min in a phosphate–glycine bath and then bathe three to five times in a phosphate–glycine buffer.
- *Inhibit non-specific reactions:* Leave for 5 min in a phosphate–ovalbumine buffer.
- *Permeabilize the resin-embedded sections:* Use 10% H_2O_2 for 10–15 min. Rinse five times for 3 min each time in the phosphate buffer.
- *Permeabilize the tissue:* Triton solution (0.1%) or saponin (0.5%), in an adapted phosphate buffer.

(b) *Labeling steps***:**

- *Incubate* the sample for 10 min to 2 h (depending on the speed of penetration into the antigen) in a solution of specific primary antibodies diluted (often 1:20) in a phosphate buffer. Dilution must be tested beforehand via classic histology.

- *Rinse:* Three to five times for 3 min each time in the phosphate (or phosphate–ovalbumine) buffer.
- *Label with the conjugate:* Secondary antibodies + gold particles diluted in the phosphate buffer for 1 h. Rinse three to five times in the phosphate buffer.
- *Fix and/or post-fix the samples*, if necessary.
- *Complete the technique*: Embedding, slicing, or contrasting, depending on the situation.

(c) *Verifications:*

- *Halt the Reaction:* This consists of incubating a control sample in the antibody solution adsorbed by the corresponding antigen in excess solution. Perform the same procedure (b) below. No labeling should appear.
- *Test the non-reactivity of the conjugate:* Perform this step on another control sample, while omitting the step of incubation in the antibody and only incubating the conjugate solution. No labeling should appear. Successive incubation baths are performed on drops deposited onto plastic film. Rinses are performed in small trays. The thin sections are carried in loops and deposited onto each drop or each tray and then picked up.

Following these guidelines, the procedure can be adapted to each sample. For more information, refer to the bibliography.

Protocol Selection

Taking into account all these considerations, there can be a wide variety of protocols. Physical techniques are always more complex to implement. Therefore, it is preferable to start with simpler protocols and then gradually introduce low-temperature steps in a classic protocol. Immunolabeling can be carried out after one of the steps of the chemical or physical preparation protocols for samples, but some chemical steps can be performed after physical steps and vice versa.

Criteria Defining the Choice

Perform immunolabeling as early as possible in the sample preparation timeline. If the antigen is directly accessible, e.g., a membrane antigen on culture cells, immunolabeling must be performed before any fixation. *Caution:* In this case, the tissue is alive, and the surface antigen can be labeled and then migrate into the cell if the reaction or rinse times before fixation are too long. Incubation and rinse time before fixation will be brief, in order to prevent this migration.

Tables 7.2 and 7.3 summarize the different protocols that can be used depending on the type of antigen to be revealed and the type of tissue.

For bulk tissues, more intense labeling will be obtained on frozen hydrated slices cut in a cryostat, or better still on ultrathin cryo-slices (i.e., the Tokuyasu technique).

Table 7.2 Protocol examples

Protocol 1	Protocol 2	Protocol 3
Removal	Removal	Removal
Immunolabeling		
Aldehyde fixation	Aldehyde fixation	Aldehyde fixation
Rinse	Rinse	Rinse
	Permeabilization[a] of the membrane if necessary	
	Immunolabeling	
Post-fixation	Post-fixation	Post-fixation
Rinse	Rinse	Rinse
Dehydration	Dehydration	Dehydration
Infiltration	Infiltration	Infiltration
Embedding	Embedding	Embedding (in LR White)
Slices	Slices	Slices
		Immunolabeling

[a]For permeabilizing the cell membrane, a solution of triton (0.1%) or saponin (0.5%) in an adequate buffer is used.

Table 7.3 Others protocol examples

Protocol 4	Protocol 5	Protocol 6
Tokuyasu method		
Rapid aldehyde pre-fixation		Rapid aldehyde pre-fixation 1 h
Infiltration in sucrose	*Ultrarapid freezing*	
Immersion freezing		
	Cryo-substitution	*Dehydration under a temperature gradient (253–293 K) (PLT)*
	Cryo-embedding (Lowicryl)	*Cryo-embedding (Lowicryl)*
	Cryo-polymerization	*Cryo-polymerization*
Slicing at low temperature	Slicing at room temperature	Slicing at room temperature
Immunolabeling	Immunolabeling	Immunolabeling
Observation	Observation	Observation

All steps in italics are performed at low temperatures.

4.2.3 Observations

This is performed on sections that either have or have not undergone uranyl or lead contrasting. Figures 7.38, 7.39, and 7.40 show different immunolabeling examples.

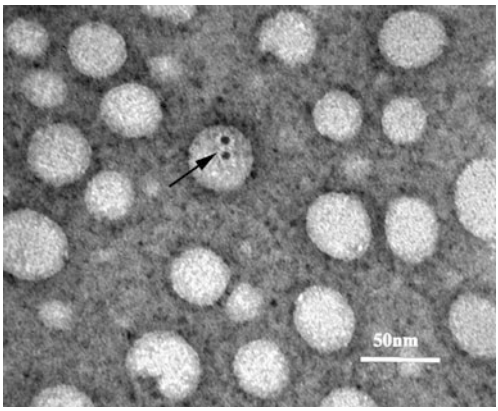

Fig. 7.38 Immunolabeling of hepatitis C viral particles in suspension. Particles are immunoprecipitated by the anti-protein antibody of the viral envelop obtained from patient serum, ultracentrifuged on a sucrose gradient, re-suspended, deposited on a grid, incubated, labeled with 5-nm gold particles, and contrasted with 1% aqueous uranyl acetate. *Arrow* indicates two gold labeling particles (*M.A Petit, CeCIL, UCB-Lyon 1*)

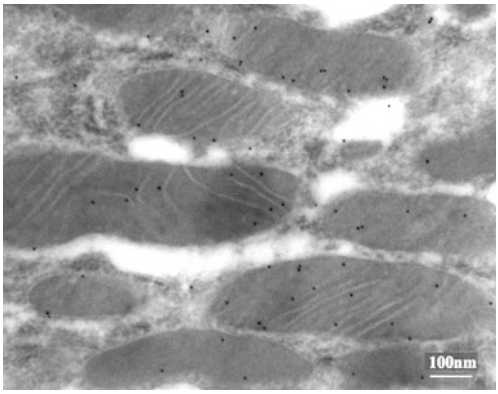

Fig. 7.39 Immunolocation of a glutamine metabolism enzyme, detected in the mitochondria of renal tubules from a mouse. Fixation with periodate–lysine–paraformaldehyde, embedding in LR White, and immunolabeling on ultrathin slices on a nickel TEM grid. There are many 50-nm gold particles (*black dots*) (*O. Levillain, CeCIL, UCB-Lyon 1*)

Gold particles are very apparent and are close to the structure possessing the antigen. If they are very small, they can be masked by the structures; if there is any doubt, it will then be easier to detect them on negatives of silver photos. Sometimes a single contrasting (usually uranyl) helps to recognize the structures and gives a better view of the gold grains.

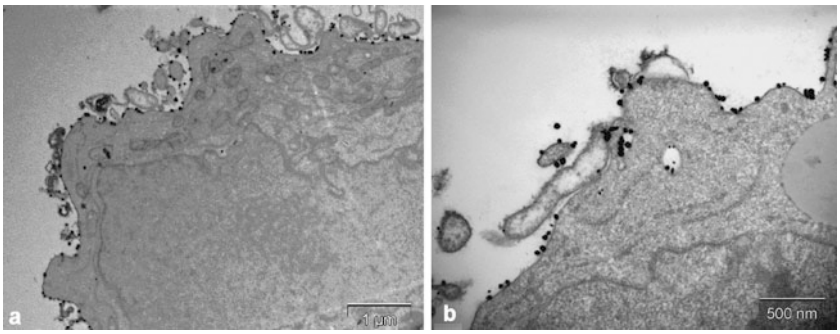

Fig. 7.40 (**a**) and (**b**) Two TEM images, at different magnifications, of isolated K562 cells on which the glycoproteins of the surface have been labeled: aldehyde fixation, labeling with 15-nm colloidal gold before embedding in Epon, double contrast (*L. Dubuisson SERCOMI, Bordeaux 2*)

If labeling seems poorly located or if present in structures where it is not expected, it could be a case of parasitic labeling. Then it will be necessary to compare the sample with controls and possibly use a statistical study in order to uncover the right information.

4.3 Variants

4.3.1 Double Labeling

Two successive labelings can be performed on the same preparation by using conjugates marked with gold particles of different sizes (Figs. 7.41 and 7.42).

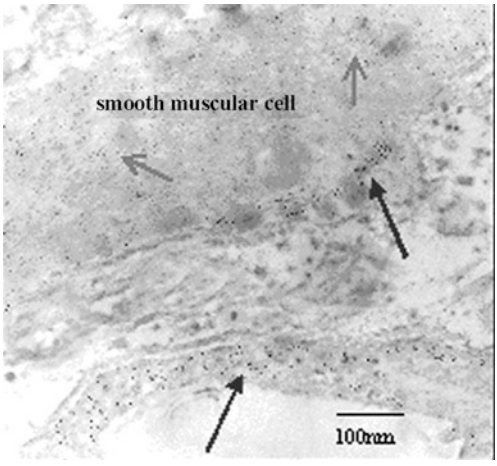

Fig. 7.41 Double immuno-location of caveolin with 10-nm gold particles (*long arrows*) in endothelial cells of a blood vessel and in the edge of a smooth muscle cell, and labeling with 5-nm gold particles (*short gray arrows*) of actin fibers in a smooth muscle. Mammary gland of a lactating ewe (*C. Longin, INRA, Jouy-en-Josas*)

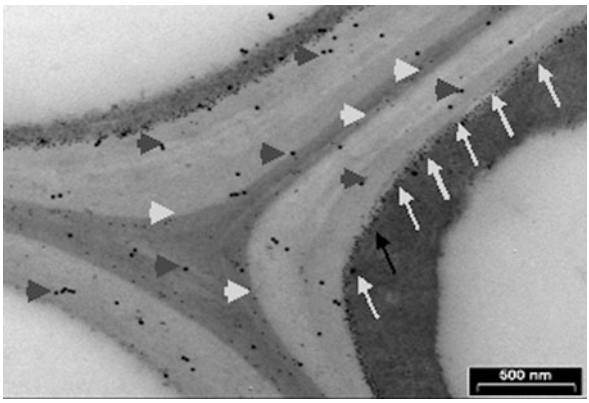

Fig. 7.42 Plant cell. Double immuno-location of two different complex polysaccharides (*short and long arrows*) in xylem walls (*A. Driouich, UMR CNRS 6037, Université Rouen*)

4.3.2 Labeling Fine Particle Materials

For fine particles, labeling can be carried out in the initial suspension. If necessary, this can be followed by a brief fixation and also by negative or positive staining, depending on the sample type (see Fig. 7.38). *Note:* In the protocols outlined in Fig. 7.38, the reaction of a specific antibody makes it possible to "sort" the corresponding particles in the patient's serum using immunoprecipitation and isolate them on increasing sucrose concentration gradients. However, this technique does not make it possible to label all isolated particles with the conjugate containing gold, because the technique must mask the reactivity of some antibodies.

4.3.3 Freeze-Fracture Labeling

This technique can also be applied to samples prepared using the freeze-fracture technique (see Pinto da Silva P., Parkinson C., Dwyer N. Freeze-fracture cytochemistry: thin sections of cells and tissues after labeling of fracture faces. J. Histochem Cytochem, 29, 917-928, 1981.)

4.4 Advantages

This technique is used to resolutely pinpoint a structure (e.g., a virus) as long as an absolutely specific membrane antibody is available (Fig. 7.38). It is also widely used to locate biological molecules within a tissue. This is called immunolocation (Figs. 7.39 and 7.40). The epitope carried by a sought-after molecule is labeled with gold particles that are easy to spot on the slice. The example in Fig. 7.39 shows that labeling is used to highlight the organelle responsible for metabolic activity. With double labeling (Figs. 7.41 and 7.42), the technique is used to locate two different molecules at the same time. By performing a kinetic study, it can be used to follow the movement from one cellular compartment to another, including its extracellular secretion. By using monoclonal antibodies, we can hope to have great

reaction specificity, i.e., marking a single category of epitope. In both cases it is always recommended to verify the specificity of the resulting reaction by using the two controls mentioned in the procedure.

4.5 Limitations

This is a delicate technique, which can be tainted by error. The specificity of the reaction must be controlled. It is sometimes difficult to find commercially available antibodies that correspond to a molecule that we want to locate.

One can nevertheless produce or manufacture a polyclonal antibody that poses the same problems of site specificity.

Antibodies are expensive, therefore reactions are performed using just one drop of them so as to reduce the amount used.

Antigens are proteins and consequently very fragile. They are denatured by osmotic fixation, by alcohols, and by polymerization at high temperature. This is why most techniques suggest labeling before fixation for isolated cells and fine particle samples, either before dehydration on cryo-slices or after embedding and polymerization in the cold under UV light. Regardless, there is always a loss of antigenicity. If there is too much of a decrease in reactive sites, immunolocation becomes hard to interpret and validate.

4.6 Artifacts

The specificity of the reaction is not always ensured; it must be verified. There must not be any gold particles on the preparation with the antibody saturated by the antigen (control 1) or using the conjugate alone (control 2). If this is not the case, the structural labels must be found and removed from the results. Specificity is disrupted by cross-reactions. The most frequent of these are due to preparation residues, particularly the aldehyde fixative and the presence of immunoglobulins. In order to prevent these cross-reactions and parasitic reactions, it is necessary to make sure that the aldehyde fixative residues are neutralized if labeling is performed after fixation, and to treat animal samples with ovalbumine, especially for culture cells in calf serum.

4.7 Type of Analysis

Structure, functional properties.

4.8 Risks

There are no specific risks related to this technique.

4.9 Conclusion

Immunolabeling is one of the most widely used techniques for tackling cell and tissue function issues in both animals and plants.

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Glossary

Advantage: main advantage over other techniques.

Anoxemia: Oxygen deprivation.

Artifact: Damage induced in the material during sample preparation that, when observed at the TEM scale, may be mistaken for the true sample structure.

Auxiliary equipment: essential or useful equipment needed to perform a specific preparation technique.

Biological material: very hydrated organic material based on C, H, O, N, resulting from processes of living organisms (proteins, DNA, RNA, viruses, liposomes, bacteria, yeasts, animal or vegetal cells, tissues).

Buffer system: aqueous solution used to maintain a constant pH value by making R^- anions available to neutralize free H^+ ions.

Bulk material: block-shaped material with 3D geometry.

Ceramic: synthetic material with an ordered structure forming the families of oxides, carbides, nitrides, borides, or silicides.

Chemical phase: compound of well-defined chemical composition and structure, which may be amorphous, mono- or poly-crystalline.

Chemical protocol: protocol for chemical preparation consisting of a series of successive steps, using various chemical compounds for the preparation of biological materials.

Chemically inert material: material that does not react with any chemical agents.

Cleavage: atomic scale fracture of some single crystal materials along a glide plane.

Compact: non-porous material.

Composite: composite material.

Composite material: complex material (usually synthetic) made up of several types of materials, whose constituents are intimately bounded, forming a new

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material with new properties (e.g., polymer resin–carbon fiber, polymer–alumina fiber).

Cryo-microtome: microtome placed in a temperature-regulated chamber, enabling the production of sections at temperatures around 233 K. The sections obtained from a frozen specimen are 10- to 100-μm thick.

Cryostat: equipment for refrigerating in a temperature range from 273 to 97 K, using a cooling circuit or a cooled gas injected through a nozzle.

Dalton (Da): the dalton is an atomic mass unit corresponding to $1.66 \cdot 10^{-27}$ kg. The molecular mass expressed in Daltons, is the sum of atomic masses of different atoms constituting a molecule. Due to the large sizes of molecules (i.e. containing from thousands to millions of atoms), the Kilodalton (kDa) unit is much more used in biology than in biochemistry.

Decoration: heavy metal (Au and Pt) deposition on various material surfaces to examine the microtopography (growth defects, surface steps, etc.).

Drawback: unavoidable and undesirable event, inherent to the preparation technique, which may induce some defects and reduce the possibilities of observation.

Ductile: Capability of a material to undergo large plastic deformation without breaking.

Electric property: insulator (ceramic, polymer, mineral, etc.) or conductor (metal, semiconductor, conducting ceramic, conducting polymer).

Embedding: operation consisting of surrounding the specimen with a resin (in order to be hardened by polymerization), to produce a block that is easier to handle, ensuring its cohesion and/or being able to select a preferential orientation. The resulting material becomes a composite material.

Evaporation: Process of evaporating metal or carbon.See evaporation gun, thermal evaporator.

- **Evaporation gun:** High vacuum system consisting of a carbon/metal cathode placed in an electric field, to deposit a thin metal film made of fine grains.
- **Thermal evaporator:** High vacuum equipment under secondary vacuum used for the evaporation of carbon or metal by resistance heating (Joule effect) or by electron-beam heating.

Extraction replica: Particle extraction from the surface of a specimen by embedding them in a thin plastic film, stripping it before shadowing, or the extraction of particles from the matrix of a multiphase material by dissolving the matrix.

FEG: Field Emission Gun

Fine particles: material made of micrometer to nanometer scale-sized particles that can be in the form of fibers, small plates, powders, spheres, tubes, isolated molecules, belonging to any type of material including biological materials.

Fracture: Breaking a material into two or more parts, as a result of a mechanical strain.

Fragility: Brittleness, ease of breaking.

Freezing: Physical method for generating ice from water solidification by lowering the temperature. The ice can be either crystalline or vitreous depending on the experimental conditions.

– **Simple freezing:** Decrease of temperature leading to a mixture of amorphous and crystalline ice.

– **Ultrafast freezing:** Abrupt freezing down to very low temperatures, leading to the formation of vitreous ice exclusively.

Hardness: Resistance to penetration (*link with Table 2.1 and Table 2.2 in Methodology volume).

– **Hard:** Material that is non-deformable or difficult to deform (can be brittle).

– **Soft:** Deformable material.

Hazard: potentiality of a piece of equipment, a substance, or a procedure to harm the health of the operator. Hazards incurred during preparation can be related to the use of chemicals, cryogenics, elevated temperatures, gases, and vacuum.

Immunolabeling: Specific labeling of a protein by an immunological type of reaction.

Immunological-type reaction: Natural antigen–antibody type of reaction which is specific to living organisms. An immunological reaction occurs between a specific site borne by a protein to be recognized (antigen) and a specific protein (antibody) produced against this antigen by an immune cell of a vertebrate.

Inclusion: is the equivalent of the embedding performed after the infiltration step.

Infiltration: Operation consisting of introducing a resin into the bulk of a specimen in order to replace the air, water, or solvent content (porous or hydrated materials, biological materials).

Labeling: Chemical reaction revealing a specific site of the material.

Limitation: Limits imposed by the preparation technique or by the material's properties, making the material difficult or impossible to be prepared by this specific technique.

Material: Metal, semiconductor, ceramic, mixed–composite, composite, biological material.

Metal: Metallic bonded material, both an electronic and thermal conductor.

Microscope support grid: Specimen support that is 3 mm (sometimes 2.3 mm) in diameter, made of metal, carbon, or polymer, with a great variety of mesh shapes

and sizes. Support grids can be coated with a continuous or a holey polymer and/or carbon film.

Microtome: Equipment for cutting thin sections (thickness order is several tens of micrometers) using metal knives.

Mineral: Natural or artificial material with precise chemical composition and ordered structure.

Mixed-composite: Category of materials made up of both mixed and composite materials.

– **Composite material:** Complex material (usually synthetic) made up of several types of materials, whose constituents are intimately bonded, forming a new material with new properties (e.g., polymer resin–carbon fiber, polymer–alumina fiber).

– **Mixed material:** Complex material made up of a mixture of several types of materials whose different phases can be easily distinguished and separated (e.g., concrete).

Molding: taking an impression of a surface.

Monolayer: Self-supported thin film with 2D geometry.

Multilayer: Material with 2D geometry composed of stacks of layers deposited on a substrate.

Multiphase material: Material composed of more than one chemical phase.

Organization of the material: Macroscopic conformation of the material, i.e., bulk, monolayer, multilayer, or fine particles.

Orientation of the specimen slice: Choice of the specimen orientation for its observation in the microscope: planar-longitudinal, transverse, particular, random, or arbitrary.

–**Planar-longitudinal orientation:** Orientation yielding a thin planar-view slice parallel to the surface of the bulk material or the multilayer. In the case of textured materials, the thin slice is parallel to the texture.

–**Transverse orientation:** Orientation along a direction perpendicular to the surface of the bulk material, the monolayer, or the multilayer, to obtain a cross section. In the case of textured material, the thin slice will be parallel to the texture.

–**Particular orientation:** Orientation other than planar-longitudinal or transverse, which is either allowed or required by the organization of the material.

–**Random orientation:** Unpredictable orientation due to the organization of the material (fine particle materials) or to the preparation technique (e.g., grinding, negative staining).

–Arbitrary orientation: There is no special requirement for the choice of specimen orientation.

Petri dish: Flat plastic or glass box that is 3–20 cm in diameter, used by biologists for cell or tissue culture.

Physical protocol: Protocol of physical preparation consisting of a series of successive steps, using low-temperature processing for biological materials preparation.

Physical state: Compact, porous material, with liquid solution.

PLT: (progressive low temperature) Technique of progressive dehydration and embedding of a biological material using a temperature gradient below 273 K.

Polymer: Organic material made up of carbon-containing chains with covalent bonding.

Porous material: Material containing numerous voids from micrometer to nanometer scale size, filled with a gas (air, etc.) or a liquid (water, solvent, etc.).

Preliminary and/or complementary preparation: Treatment made before or after the thin slice preparation technique**,** which may be imposed by the type, organization, or physical state of the material.

Préparation: technique leading to a thin or ultrathin slice.

Replica: thin film obtained after the molding of the surface topography of a material, resulting from different stages: Fracture, molding, shadowing, mold-shadowing.

Resistant: Resistant to plastic deformation up to the breaking point.

Risk: Potential consequences for the operator exposed to a hazard (see hazard).

Semiconductor: Non-conducting material**,** which becomes conductive through the introduction of impurities or oxygen vacancies in the case of oxides.

Single-phase material: Material composed of a single chemical phase.

Slice: A thick object that is opaque electrons (thickness much larger than 200 nm).

–Thin slice: Object prepared by thinning down to electron transparency in order to be observable in the electron microscope. Distinction is made between thin slice (less than 100-nm thick) and ultrathin slice (less than 50-nm thick).

–Ultrathin slice: See thin slice.

Solution: Result of the dissolution of a chemical substance in a solvent.

Specimen holder of the microscope: Housing in the microscope specimen stage, allowing the positioning and support of the specimen in the TEM.

–Specimen holder: Support for the specimen, allowing its insertion into the preparation equipment.

–Specimen stage: Support on which the specimen holder is fixed, allowing movements in the microscope or into the preparation equipment.

Sputter coater: Equipment for the deposition of metal films, under primary vacuum, using a plasma discharge (Ar^+, etc.). If the polarity is reversed, the specimen surface can be sputter-cleaned.

Sublimation: Direct change from solid to gaseous state.

Substitution: Replacement of a liquid phase by another liquid phase, without changing the organization of the material.

Support washer: 3-mm-diameter ring used instead of a microscope support grid.

Suspension: Liquid medium made of particles dispersed in a liquid (water, oil, solvent, etc.).

Thin film: Two-dimensional geometry material of small thickness deposited on a substrate.

Thin or ultrathin section: See thin slice.

Type of analysis:

Topography: Study of the surface relief and unevenness, bright field, etc.: SEM, TEM-STEM.

Structure: Study of the arrangement of the matter on different scales, various constituents [bright field (BF), dark field (DF), high resolution (HREM), chemical contrast (*Z*-contrast), TEM-STEM mode, annular dark field, cryo-microscopy, tomography, cryo-tomography].

Crystal defects: Study of the structural defects in crystalline material: 0D point defects (vacancies and interstitials), 1D linear defects (dislocations), 2D planar defects (grain boundaries and interfaces), 3D defects (precipitates, cavities, etc.) [bright field, dark field, weak beam, large angle convergent electron diffraction (LACBED), high resolution, annular dark field, *Z*-contrast].

Crystallography: Study of the crystal by electron diffraction [selected area electron diffraction (SAED), convergent beam electron diffraction (CBED), large angle convergent beam electron diffraction (LACBED), micro- and nano-diffraction (μ-diffraction)].

Chemical composition: Analysis of the elemental chemical composition by X-ray spectrometry (EDS) and by electron energy loss spectroscopy (EELS).

Chemical bond: Study of chemical bonds by electron energy loss spectroscopy; study of the chemical environment of atoms by studying the near and extended fine structure of absorption thresholds (EELS, PEELS, ELNES, EXELFS).

Properties: Study of the material's properties.

Mechanical properties: Study of the properties by in situ microscopy, strain measurements by HREM, CBED, and LACBED.

Optical, electric, electronic, and magnetic properties: In situ measurements, plasmon analysis, EELS analysis, holography, Lorentz microscopy.

Functional properties: Localization of functional sites by specific markers (bright field, annular dark field, *Z*-contrast).

EBSD: Electron Backscattered Diffraction analysis is used to analyse the crystallographic orientation of a SEM or TEM specimen surface.

EBIC: Electron Beam Induced Current Imaging technique used in SEM and TEM microscopes.

EDS: Energy Dispersive Spectrometry analysis used commonly for chemical composition of all types of materials.

EELS: Electron energy loss spectroscopy: sequential analysis technique for studying the chemical composition, chemical bonds, and the chemical environment of an atom.

Lorentz Microscopy: magnetic domain imaging mode is performed in conventional TEM.

PEELS: Parallel electron energy loss spectroscopy: technique of analysis in parallel mode for studying the chemical composition, chemical bonds, and the chemical environment of an atom.

Plasmons: Collective oscillations of a free electron gas which enable local dielectric property analysis. These quantified plasma oscillations behave as quasiparticles.

Holography: Imaging technique for electric and magnetic local physical properties.

CTEM: Conventionnal Transmission Electron Microscopy is performed in parallel incident electron beam.

Dark Field (DF): CTEM imaging mode used for structural defects analysis.

Bright Field (BF): CTEM imaging mode used for structure analysis.

Weak Beam: conventionnal Dark Field TEM imaging mode used in CTEM, for structural defects analysis.

HAADF: high angle annular dark field performed in STEM, is a Z-contrast atomic level chemical imaging mode.

HRTEM: High Resolution Transmission Electron Microscopy imaging is performed in TEM mode.

TEM: transmission electron microscope

STEM: scanning transmission electron microscope

SAED: selected area electron diffraction

Dedicated STEM: Scanning Transmission Electron Microscope

CBED: convergent beam electron diffraction

LACBED: large angle convergent beam electron diffraction

Microdiffraction: Low angle convergent electron diffraction.

Nanodiffraction: parallel electron diffraction mode using a nanometer size electron probe.

ELNES: energy loss near edge structure: technique to analyze the fine structure of the losses near the absorption threshold of an atom. It allows the analysis of bonds and the chemical atom.

EXELFS: extended energy loss fine structure: technique to analyze the fine structure of the absorption threshold covering the range from the threshold to distant losses. This technique provides information on the structural organization around an atom in a crystalline solid, as deduced from the oscillations caused by the periodicity of the crystal network.

Type of material: metal, semiconductor, ceramic, mineral, polymer, biological material, mixed–composite material.

Ultramicrotome: equipment for preparing thin or ultrathin sections (less than 100-nm thick using diamond knives).

Ultrathin slice: Specimen of less than 50nm thick, to be observable in the TEM, for high resolution analysis, EELS, etc..

Variant: modification of the technique for some applications or some types of materials.

Vector liquid: liquid with three components (fixative agent, buffer system, and osmolarity regulator agent) used for the chemical fixation techniques.

Vibratome: equipment producing 5- to 150-μm sections in a fresh tissue, using a razor blade.

Vitreous ice: amorphous ice with no evidence of any crystalline features on the electron microscope scale.

With liquid solution: material containing a liquid phase or material dissolved in a liquid.

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