

# TECH-NOTES

## USING MICROSTRUCTURAL ANALYSIS TO SOLVE PRACTICAL PROBLEMS

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## Specimen Preparation of Bones, Tissues and Other Similar Materials

### Introduction

In 1849 when Henry Sorby, the father of petrography, prepared a thin section, he may have not envisioned that one day his technique would be used to analyze many diverse materials such as bones and tissues. Sorby showed to the scientific community that by using simple techniques, one could reveal the microstructure of materials so that it could be observed with a microscope. Although Sorby's method of preparation was crude compared to the method used today, his basic technique for preparing and examining a specimen with transmitted or reflected light has remained fairly similar.

In the last decade, considerable improvements have been made to repair damaged bones and tissue. Devices such as hip implants and stents (a narrow metal or plastic tube inserted into the artery to keep a formerly blocked passageway open) are being used extensively to repair and correct problems, which were difficult to correct in the past.

Use of surgical implants in the body has greatly improved the quality of life by correcting and preventing deformities, reducing pain, and improving body functions. With the aging population growing, there is a rapid increase in the use of implants to improve and correct body functions and repair damage such as hip replacement.

It is necessary for a successful implantation that the bone or the tissue accepts the implant and does not reject the intrusion. Surgical implants are exposed to biochemical environment of the body, which can cause degradation of the implants. Because biocompatibility of the implant in the body is a major concern and is the main reason for microscopic examination, it is important that any method that is used to prepare a bone or tissue specimen must not alter or hide the true microstructure, which can confound the analysis.

Properties of bones and tissues differ greatly from those of implant materials such as metal, ceramic, and polymers. Bone is spongy and can regenerate thus can change its volume with time. Implants on the other

hand are hard and do not change in volume and can degrade in the body, causing failure.

The dissimilarity of the material that is a hard implant in contact with a much softer bone or tissue makes specimen preparation rather difficult. The technique employed to prepare biomaterial specimen for microscopic analysis must prevent damage to the bone or the tissue and at the same time must maintain the integrity of the implant. The bone and implant interface, which shows if the implant has been accepted or rejected by the body must be preserved and not altered or damaged by poor preparation practice.



Figure 1. Microstructure of a bone thin section in transmitted light. Dark area is the implant.

For micro-radiography it is important to have a parallel and smooth thin section so that the evaluation of metabolic bone diseases and osteon density can be easily performed. Specimens for micro-radiography do not require as good a surface quality as for microscopic evaluation. In most cases a good saw cut surface produced by a precision diamond saw such as one of the ISOMET® saws, is sufficient for radiographic purpose.

Generally, the paraffin method is used to prepare de-calcified bones and soft tissues where the specimen is sliced with a microtome. However, microtome cannot be effectively used for bone or tissue that con-



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tains an implant, because the microtome knife can be easily damaged by the implant. A damaged knife can cause tearing of the tissues thus damaging the specimen. Also in some cases such as when sectioning de-calcified bone, the microtome can cause folds, shrinkage, or micro cracks, which can make microscopic examination difficult.



Figure 2. ISOMET® Low Speed Saw used for sectioning biomedical materials

Bones and tissues are studied by two different methods. Thin sections, which are used for micro-radiography and for observing with transmitted light, and polished, bulk specimens where the top surface is polished and the specimen is observed with incident light (reflected light). Thin sections are much more common and provide considerably more information than bulk specimens; therefore they will be discussed in detail. A polished thin section containing an implant can be used for both transmitted and reflected light examination. The implant, which is opaque, can be examined by reflected light for features such as porosity, grain size and corrosion. The bone or the tissue, which are transparent, can be examined in transmitted light. Cellular features, such as osteons and haversian canals, show their structural details more clearly in a thin section and features, such as pores, coatings (HA) and corrosion in metallic implants are better observed with a polished section.

The method generally used for preparing thin sections of bone or tissue consists of sectioning the specimen with a precision saw, embedding it in a castable resin under vacuum and then grinding and polishing it. In some cases, it may be desirable to embed the specimen first and then section it with a saw. This is done for weak, fragile or small specimens, which will otherwise break or are too small to be held securely in a chuck during sectioning. Once the specimen has been sectioned, one side of the specimen is ground to remove the saw marks and expose the surface if the specimen was encapsulated. It is then attached to a slide using epoxy or any other suitable adhesive. The specimen is re-sectioned to decrease its thickness to save grinding time and then ground and polished to reveal cellular details.

For micro radiography a specimen approximately 80-100 $\mu$ m thick is suitable; but for observing with transmitted light a thin section should be between 20 and 50 $\mu$ m in thickness, or it is ground until the cellular details become clear for observation. To observe tooth enamel, sections of approximately 40 $\mu$ m thick are reported to be better for examining Wilson bands for anthropological work.

When preparing a thin section, it is advisable to maintain the top and the bottom surface of the specimen parallel. A wedge-

shape specimen is not suitable for microscopic examination or for micro-radiography because it may not reveal the micro-structure clearly over the entire surface.

### Considerations in Sectioning the Specimens

The first step in preparing a histological specimen is sectioning (cutting), which must be performed with care so as to avoid damaging the tissue or the tissue-implant interface.

To section biomaterials, several precision diamond wafering saws are available. The selection of the saw depends upon the following.

- Size of the specimen to be cut
- Size of the implant
- Cutting speed
- Number of specimen required to be cut in a day
- Manual or automatic serial sectioning
- Manual or automatic operation of the saw

The following is a brief description of the Buehler saws available for sectioning biomaterials. All saws are tabletop units. Sectioning parameters such as the speed of the blade, force applied for sectioning, and the specimen thickness can be controlled.

ISOMET® Low Speed saw has been designed to section small specimens precisely. Cutting times are much longer than those with higher speed saws. Maximum speed is 300 R.P.M. and the maximum blade size that can be used is 5 inches (127mm). A precision micrometer, which is also available with metric graduations, controls the thickness of the cut.

ISOMET® 1000 has large cutting capacity with a metric or inch digital display. Maximum speed is 1000 R.P.M. Maximum blade size is 7 inches (178mm). It has a built-in blade-dressing device and the cutting compartment is enclosed.

ISOMET® 4000 has a large cutting capacity. Maximum speed is 5000 R.P.M. The SMARTCUT system of the saw detects if the cutting force is excessive, and automatically reduces the force to prevent damage to the specimen or to the blade. A precision digital micrometer controls the thickness of the cut. The cutting chamber has a hood. A digital panel controls the cutting parameters such as speed, force and feed rate. Maximum blade size is 7 inches (178mm). The control panel is capable of displaying the cutting parameters in several languages.

ISOMET® 5000 is a fully automatic linear feed precision saw. It is similar to the Isomet 4000; however, it is capable of automatic serial sectioning of specimen to a precise thickness. This is achieved by using an automatic linear feed motor, which advances the specimen for repeated cuts of desired thickness. The thickness, speed, feed rate and other parameters are adjustable. The saw is ideal for serial sectioning bones or teeth where several thin sections can be used for mapping the specimen.

### Selection of a proper blade

Buehler's wafering blades are designed to perform specific functions. General-purpose blades are designed to cut different types of materials in a reasonable amount of time. However, the cut surface may not be as good as produced by a blade that is designed to cut with very little deformation in the surface, such as the series 10 blades. However, a blade that is

designed to cut with little deformation will take much longer to cut the surface. On the other hand a blade that is designed to cut rapidly will most likely produce less satisfactory surface. In other word no one blade will do every thing that is cut very rapidly and at the same time produce an excellent surface. To cut bones with large implants, one may have to compromise between surface finish and cutting time.

To cut brittle materials such as hydroxyapatite coating a LC10 blade, which is designed to cut brittle materials will be a better choice.



Figure 3. ISOMET® 5000. A fully automatic saw capable of serial sectioning materials very precisely.

### ISOCUT® Wafering Blades

ISOCUT® blades have a synthetic abrasive and are especially designed to cut ferrous materials, nickel, cobalt, and super alloys. Generally, diamond blades do not cut these materials as well. These blades can significantly reduce the cutting time for these materials.

### Kerf Loss and Preventing Blade Damage

Kerf loss is the amount of material lost due to the thickness of the blade as it slices through a specimen. When processing biomaterials, sometimes only a small quantity of material is available; therefore, preserving the material is of prime impor-

tance. To minimize the kerf loss, a thin blade is selected. For stability, a thin blade requires large flanges for support so that the blade does not bend or wobble during cutting. Because a large flange limits the size of the specimen that can be sectioned with the blade, people try to use the smallest flange available to increase exposure of the cutting edge. However, when a small flange is used, it does not provide proper support to a thin blade and this lack of support can result in bending and damage to the blade. A large blade, on the other hand, is generally thicker; therefore it does not require as much support as a thin blade and can be used with smaller flanges. It is therefore suggested to use as large a blade as possible to minimize damaging the blade. When a thin blade must be used, such as the 3 inch (176mm) diameter blade, (0.006 inches or 0.15mm) in thickness, do not apply excessive force for sectioning as this may damage the blade.

### Gang Cutting

Gang cutting is a method that can be used for sectioning small bones, teeth and tissues, where two blades are used simultaneously rather than one blade, with a spacer in between them. The thickness of the spacer determines the thickness of the specimen that will be cut. Although the gang-cutting method can produce specimens of precise thickness, because two blades are used simultaneously, there is considerable increase in the cutting time.

### Disadvantage of Cutting a Very Thin Bone or Tissue Section for Making Thin-Sections

Although precision saws are capable of cutting very thin specimens of bones and tissues, there is a tendency for a thinly cut specimen to curl. Unless the tissues are to be used in the as-cut condition and do not have to be cemented to a slide, it is advisable to cut them thick enough so that they can be held by hand and ground easily. Also, when a specimen is attached to a slide, pressure is applied to keep the specimen flat against the slide during the cure cycle. If a specimen is very thin the mounting material can seep from underneath the specimen and can come in contact with the device that is used for applying the pressure and stick to it, causing a problem.

## EPO-THIN® Low Viscosity Epoxy



- Free-flowing, specially formulated epoxy that fills holes and voids where other mounting materials fail
- Produces clear, bubble-free mounts, allowing full view of the embedded sample
  - Low cure temperature, 80 °F (27 °C)
  - 9 hour cure time
  - Ideal as a thin section mounting adhesive



EPO-THIN® is free flowing for filling small holes and voids.



## Impregnation and Encapsulation (Embedding)

The most common medium to embed bones and tissues is methyl-methacrylate that is used by most institutions. However, embedding in methyl methacrylate as practiced in hospitals requires several steps and can take several days. Epoxies on the other hand, if they can be used, cure within hours. Epoxies do not take the stains as well as methyl-methacrylate. If an epoxy can be used for embedding, vacuum impregnation and encapsulation can be accomplished simultaneously by using our vacuum impregnation equipment. Vacuum impregnation assists in filling the pores and voids in histology specimens so that they do not tear or break apart during preparation. To embed and encapsulate histology specimens such as bones and tissues, a thin embedding medium is more suitable. For this reason EPO-THIN<sup>®</sup>, a low-viscosity epoxy is more desirable than other epoxies.

To impregnate and embed the specimen, place it in a mold, such as the SAMPL-KUP<sup>®</sup> Mold. Place the SAMPL-KUP mold and the paper cup containing the epoxy, in the vacuum chamber. Turn the vacuum pump on to evacuate the chamber, which draws air from the pores of the specimen and facilitates filling the pores with epoxy. When the proper vacuum has been attained, tilt the epoxy cup and pour the epoxy into the mold. Keep the mold under vacuum for 2-3 minutes and then slowly release the vacuum allowing air to enter the chamber. This forces the embedding medium into the pores. The specimen should not be left under vacuum during the curing cycle. Once the epoxy has cured, the specimens can be sectioned, ground, and attached to a slide; or if a bulk specimen for reflected light microscopy has to be prepared, it can be ground and polished.



Figure 4. VACUUM IMPREGNATION device used to fill pores in bones with epoxy. Several specimens can be impregnated simultaneously.

## Preparation Procedure for Thin Sections

When preparing a thin section it is useful to maintain the top and the bottom surface parallel to each other, especially if the specimen is going to be used for micro-radiography or for examining with a transmitted light microscope. To maintain the two surfaces parallel, the specimens can be prepared with semi-automatic machines such as the PETRO-THIN<sup>®</sup> thin sectioning system, or by hand on a rotating wheel using special holders. PETRO-THIN system is designed to prepare thin sections of precise thickness. Its use assures consistent results. The PETRO-THIN is designed to both cut and grind a specimen. However, for medical application the specimens, especially if it has an implant, should be cut with a precision saw first and then ground with the PETRO-THIN system. Cutting the specimen with one of the ISOMET saws will not only conserve the specimen but also minimize tissue damage. The thick blade required for PETRO-THIN can cause damage to the implant and the tissue. Once cut with the precision saw, it can then be ground with the PETRO-THIN system to obtain a specimen of precise and uniform thickness.

If the specimen is prepared by hand without the aid of a special holder, the following problems are encountered.

- Lengthy preparation procedure.
- Non-uniform results.
- Difficulty in maintaining uniform thickness

To minimize some of the problems mentioned above, when a specimen is to be prepared by hand, using a holder such as the Histologic Precision Grinding Fixture assists in obtaining a specimen of very uniform thickness. The fixture has carbide stops, that prevent the specimen from being over ground and helps in obtaining a specimen of uniform and of desired thickness. The fixture is designed for slides that are 27 x 46mm in size and cannot accommodate slides of other sizes.

For histological thin sections a general preparation procedure is as follows.

### Procedure for Making Thin Sections for Transmitted Light Microscope

1. Cut the specimen approximately 2mm thin section, preferably using a precision diamond saw. Wash it thoroughly dry it, and embed in EPO-THIN resin under vacuum.
2. When cured, grind one side with 600 and 800 grit CARBIMET<sup>®</sup> silicon carbide abrasive paper until the entire surface is exposed and is flat.
3. Clean the specimen and the slide thoroughly and dry. Attach the groundside to the slide using epoxy or cyanoacrylate glue. Cyanoacrylate must be used very carefully because it cures extremely rapidly and can bond fingers to the slide if not handled properly. Cyanoacrylate glue should be used with plastic slides. When using glass slide, grinding the surface with 600 grit abrasive before attaching the specimen, will improve the adhesion between slide and the specimen.
4. When using epoxy to obtain a good, uniform thin bond, place the slide in PETROBOND<sup>™</sup> bonding fixture to apply pressure while curing, or apply pressure by other means. However, make sure that the epoxy does not come in contact with any part of the bonding jig. Place the PETROBOND jig on a hot plate at 113-122 °F (45-50 °C) for 2-3 hours. Heat will accelerate the cure cycle. If heat is not applied, the epoxy may take 24 hours or longer to cure.
5. When the epoxy has cured completely and cannot be indented by a fingernail, remove and cool the slide room temperature.
6. The specimen can now be re-sectioned with a saw to reduce its thickness or ground with the PETRO-THIN system. If it has to be ground by hand use coarser abrasives such as 320-400 grit paper until approximately 125µm in thickness. Reduce the thickness further by grinding with finer abrasive such as 600 and 800 grit papers until approximately 20-50µm in thickness or until the cellular details become clear. One has to be extremely careful when grinding a specimen less than 50µm in thickness.
7. Polish on TEXMET<sup>®</sup> 1000 or Nylon polishing cloth using 3µm METADI<sup>®</sup> Diamond Paste. Use METADI<sup>®</sup> Fluid as the lubricant.
8. If required the specimen can be final polished on MASTERTEX<sup>®</sup> or MICROCLOTH<sup>®</sup> polishing cloths with MASTERPREP<sup>™</sup> or MASTERMET<sup>®</sup> abrasive suspension for a short time to remove the scratches.

Although thin sections can be observed with a transmitted light microscope without polishing, the advantage of polishing is that it allows observations to be made in transmitted as well as in reflected light. To polish stents use TEXMET 1000 with 0.3 $\mu$ m alumina for rough polishing and use MASTERMET abrasive suspension with MICROCLOTH polishing cloth for a short time for final polishing to remove the scratches.

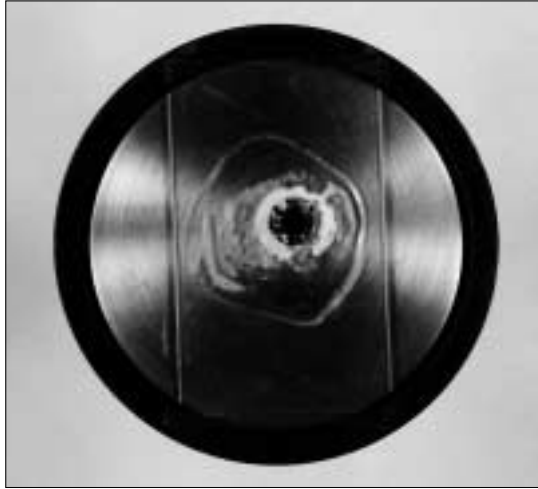


Figure 5. Histologic precision grinding fixture used to prepare thin sections. Fixture has carbide stops to prevent the specimen from being over ground, and to obtain a specimen of uniform thickness.

**Note:** It is difficult to recommend an exact size abrasive for the first grinding step because the selection of the abrasive grit will depend upon the size and thickness of the specimen, whether it has a implant or not, the size of the implant and the starting surface finish of the specimen. Therefore, use the procedure as a guide and modify it to meet different conditions.

Adhesion of the specimen to the glass slide can be improved by grinding one side of the glass slide with 400 or 600 grit abrasives.

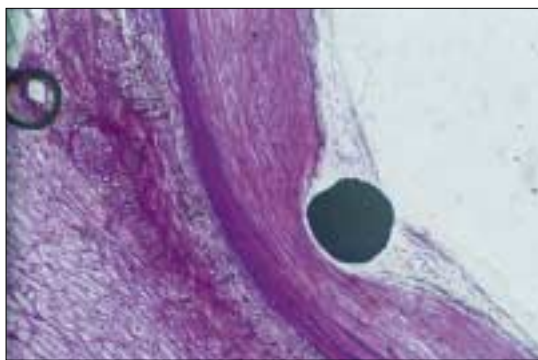


Figure 6. Thin section of a stented artery showing tissue stent interface.

To prepare bulk specimens for reflected light analysis, use the following method as a general preparation procedure for bone, bone with implants, teeth and similar materials. Preparation times and the size of abrasives used for grinding and polishing the specimen may vary depending on the size of the specimen, size of implant, and the embedding material. A rat bone will take far less time than a human bone and may require only fine abrasives for grinding.

#### Polishing Procedure for Bone and Teeth for Examination in Reflected Light

1. Section the tooth in half to minimize the grinding time. The bone can be cut to the thickness desired.
2. Embed the tooth in EPOXICURE™ resin. Bone should be vacuum impregnated and embedded in EPO-THIN or EPOXICURE resins.
3. Grind with 320, 400 and 600 grit abrasive paper. Large implants will require coarser abrasives such as 180-240 grit.
4. Rough polish with 6 micron METADI diamond paste on TEXMET 1000 polishing pad until grinding scratches are removed.
5. To final polish use MASTERPREP alumina suspension on MASTERTEX or MICROCLOTH polishing cloths until scratches are removed. Polish for as short a time as possible to minimize relief formation at the tissue implant interface.

For teeth both radiographic as well as microscopic methods are used to study features such as density, porosity, hardness and the thickness of the enamel. Anthropology departments also utilize these techniques to analyze enamel defects and to determine infection, malnutrition, fever and diet of prehistoric populations.

Generally radiography does not require any further preparation other than a good quality cut surface and that can be achieved by using a wafering saw such, as one of the ISOMET saws. To determine hardness

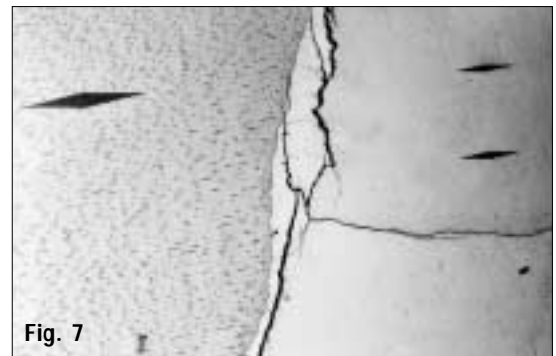


Fig. 7

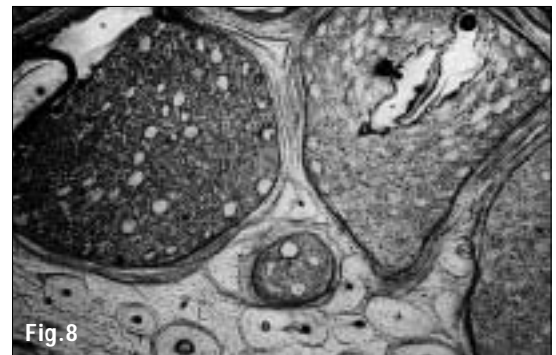


Fig. 8

Figure 7. Microhardness indentations in a tooth. The large indent shows that the dentine is much softer than the enamel (smaller indent). Figure 8. Thin section of a canine femur showing structure of osteons and haversian canals.

of the enamel, the specimen is embedded in epoxy or acrylic and the surface is polished. A micro-hardness tester can then be used for hardness measurements. The photomicrograph shows indentations, which can be used to determine the relative hardness of the enamel and the dentine.

When examining teeth, the mounting material must surround the specimen tightly and should not have a gap, because the brittle enamel has a tendency to chip during preparation.

### Other Applications of Materials Analysis

Apart from routine biological applications, microscopy can be used in other medical applications such as in the pharmaceutical industry. Other applications include, examining the integrity and the wall thickness of drug delivery bags used in the hospitals. Many drugs require special protection from moisture, light, and air and have multi-layers, which can be examined with the aid of a microscope to check the integrity of the layers and the film thickness.

Materials analysis in the medical field can be used to understand biomaterials and how they interact with the human body. By understanding the reaction of the body to biomaterials, such as implants, we can better diagnose the problem and find a method to correct it, thus reducing pain and suffering.

### Summary

The purpose of this TECHNOTE is to explain the preparation procedures and to describe a simple method of producing high quality specimens of biomaterials. It is also intended that the histotechnologists understand the basic process of specimen preparation as well as an understanding of the equipment available for preparing the specimens. An understanding of the process and the equipment will help in examining the anatomic features of bone, tissues, teeth and other biological materials with ease.

**Question:** When preparing a thin section manually, it is difficult to hold the glass slide, if a thin section holder is not available. What can I do to prevent them from slipping out of my hand?

**Answer:** Cut a piece of No 30-7508 MET-GRIP® liner (a double stick pad) slightly smaller than the slide. Peel the backing paper of the MET-GRIP liner and attach it to the back of the glass slide. Peel the backing paper from the other side and place fingers on the sticky surface. This will prevent slipping of the glass slide.

**Question:** How can I hold a glass slide when using the VIBROMET® Vibratory Polisher?

**Answer:** Insert the No. 69-1580 Thin Section Attachment into a 1½" (40mm) VIBROMET holder

and tighten the set screw. Apply a thin coating of oil to the thin section holder, insert the slide and place it in the VIBROMET.

**Question:** How can I prevent abrasive papers and polishing cloths from sticking to the platen?

**Answer:** Clean and dry the platen. Turn the platen on and apply a light coating of No. 20-8185 Release Agent to the platen surface. After the liquid has evaporated, remove the excess from the platen surface by wiping it lightly with a paper towel. Too much release agent will prevent the abrasive paper or the polishing cloth from adhering to the platen.

**If you have a question that you'd like to see answered, or a tip that you feel would benefit our readers, please write, call or fax to:**

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