Correlative Light and Backscattered Electron Microscopy of Bone—Part I: Specimen Preparation Methods

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Summary: A method for preparing nondecified bone and tooth specimens for imaging by both light microscopy (LM) and backscattered electron microscopy in the scanning electron microscope (BSE-SEM) is presented. Bone blocks are embedded in a polymethylmethacrylate (PMMA) mixture and mounted on glass slides using components of a light-cured dental adhesive system. This method of slide preparation allows correlative studies to be carried out between different microscopy modes, using the same histologic section. It also represents a large time savings relative to other mounting methods whose media require long cure times.

Key words: correlative studies, light microscopy, scanning electron microscopy, backscattered electron microscopy, light cure dental adhesives

Introduction

This paper concerns the development of preparation techniques that allow specimens to be analyzed by both light (LM) and scanning electron microscopy (SEM). Correlation of information obtained from different imaging modes is important in the study of mineralized tissues, as there are aspects of the morphology and material properties that can best be appreciated by examining the specimen using multiple microscopy sources.

Many of the resins and preparation techniques that are suitable for LM are incompatible with the electron beam. They are often mechanically unstable and therefore become physically damaged (i.e., vaporize) after continuous exposures, and may be too tacky or soft (Witcomb 1981). Some epoxy resins have the tendency to accumulate diamond in the polishing process, leading to difficulties in LM and backscattered electron (BSE)-SEM imaging. Similarly, many electron microscopy (EM) tolerant resins are not suitable for use in thin section preparations as they are too brittle to allow for sectioning and polishing of the specimen, and many are not optically clear (Witcomb 1981).

Despite these difficulties, it would be of interest to develop methods that would allow for correlative LM and SEM, as such an approach would be important to the understanding of biological structure and function (Wetzel and Albrecht 1989). Techniques have been developed to correlate LM and SEM on samples adjacent to one another (each prepared specially for LM or SEM imaging), although such methods do not allow for location of exact fields of view between the two imaging modalities (see Lynn 1975, see Wouters 1987 for review). Many correlative techniques were developed for examining localized areas of structure at high resolution, such as a cell or cell group (Kristof 1997). Specimens would be viewed by LM to find a region of interest, then reprepared for EM for higher resolution imaging by means of re-embedding and resectioning samples (Kristof 1997; Oates et al. 1997; see Wouters et al. 1987 for review). These methods are restrictive in that specimens cannot be re-imaged by LM once re-prepared for SEM. Some researchers have successfully produced sections that could be viewed in both LM and SEM without re-preparation through polychromatic staining and cytochemical techniques (Pasquinelli et al. 1985).

Existing correlative LM and SEM techniques are not directly applicable to our applications using ground bone sections. Most existing correlative research in mineralized tissue biology has been done using adjacent sections (i.e. Riggs et al. 1993), thereby limiting the ability for direct comparisons, or involve re-preparation steps that eliminate the possibility of returning to other imaging modes (Skedros et al. 1996). We therefore developed a new technique of section preparation, using embedded bone blocks prepared as mounted sections and using light-cured dental adhesives as

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mounting media. This method allows for rapid preparation of specimens that may be examined in both LM and SEM in multiple imaging sessions.

Materials and Methods

The specimen preparation methods described below were tested on a variety of nondecalcified bone specimens from several animal species. The resulting sections were imaged by a variety of microscopy modes including transmitted light, polarized light, fluorescence, SEM, and BSE microscopy.

Embedding

Bone blocks, each approximately 0.5 cm in thickness, are cleaned of adhering soft tissue, save osteoid, by a 1% enzymatic detergent solution (Terg-a-zyme, Alconox, N.Y.) with occasional ultrasonication at 50°C for 7 to 14 days. Cleaned specimens are then dehydrated in a series of graded alcohols under occasional ultrasonication and vacuum, then refluired for 7 to 14 days in heptane-isopropanol using a soxhlet apparatus.

Following this dehydrating and defatting procedure, we prepared specimens for embedding, using a variety of alternative procedures in an attempt to improve the resulting embedding of the bone and the quality of our final section. These experiments are presented here.

Chemical dehydration: Specimens were transferred through two changes of 100% methanol for 12 h each change, then soaked in two changes of xylene for 1 h each change (both under occasional ultrasonication and vacuum). They were then put through three changes of polymethylmethacrylate (PMMA) and styrene (using 0.5 gm/500 ml azobisisobutyronitrile as catalyst) for 24 h each change according to procedures described by Boyd (1984). Specimens were intermittently vacuumed during each change of PMMA, including the third change in which the specimen was allowed to polymerize at room temperature.

Styrene-sulfonate: In an effort to improve the affinity between the organic component of bone and the PMMA mixture, we subjected specimens to 0.2 molar solution of 4-styrenesulfonic sodium salt (Aldrich Chemical #32, 859-6, Milwaukee, Wisc.), following soxhlet reflux. The specimens remained in the solution for 12 h, followed by rinsing in distilled water to remove the salt. Specimens were then dehydrated in a series of graded alcohols followed by methanol, then xylene, before being transferred to PMMA changes as above.

SpeedVac: Specimens were dehydrated with methanol as above (experiment 1) and placed into a Savant SC200 SpeedVac (Savant Instruments, Inc., Holbrook, N.Y.) for 48 h. This method dehydrates the specimen through centrifugation under vacuum. Following the SpeedVac, the specimen was placed into xylene before being transferred to PMMA changes as above (experiment 1).

Critical point dryer: A test specimen, chemically dehydrated to methanol, was placed into an Autosamdr 810 critical point dryer (Tousimis, Rockville, Md.). The critical point dryer (CPD) dehydrates the specimen by replacing water in the specimen with liquid CO₂ using methanol as an intermediate medium. Following critical point drying, the specimen was placed into xylene before being transferred to PMMA changes as above.

Surface Preparation of Bone Blocks

Once embedding is complete, polymerized blocks are ground to the section level on one side using a series of graded emery papers to 1200 grit on a Handimet II (Buehler Ltd., Lake Bluff, Ill.). With the ground face down, the block is placed onto a thin film of acetonitrile for 5 min. Acetonitrile, while being miscible in water and thus compatible with wetting procedures (below), also solubilizes PMMA. This procedure provides for some surface topography of the embedding medium, needed to enhance the adhesion of the block to the glass slide. A small amount (the amount determined by the size of the specimen) of Dentin Activator (Parkell, Farmingdale, N.Y.) is brushed onto the block (covering only the area of bone itself), left for 10 s, then rinsed and air dried. The dentin activator contains ferric chloride which minimally etches the bone surface to face the glass slide in order to enhance its adhesion to the mounting medium.

Preparation of Glass Slide Surface

A histologic slide is etched with 20% hydrofluoric acid for 3 to 4 min, thoroughly rinsed, and air dried. The slide is then treated with a silane porcelain primer (Bisco, Itasca, Ill.) for 30 s and air dried. These treatments enhance the bonding between the glass slide, the adhesive resin, and the embedded block.

Mounting of Specimens

The block is mounted on a glass slide using components of the Dentsply Prime & Bond 2.1 dual cure and Probond light cure adhesive systems (Dentsply International Inc., York, Penn.). First, a primer, made of a 50:50 mixture of Prime and Bond 2.1 light cured adhesive and self cure adhesive, is applied to the block. Small specimens may be placed into a pool of the mixture for 1 min and then allowed to air dry. For larger specimens, the polished face of the block should be brushed with a minimum of five coats of the primer mixture, applied in quick succession. After air drying, the primed surface should be polymerized for 30 s using a visible light curing gun.

With the primed and polymerized surface face down, the block is pressed onto the glass slide in a pool of Pro Bond adhesive and light cured for 1 to 2 min (adherence occurs within several seconds, but continued light curing raises the proportion of cured resin near to final values). The addition of a small amount of Prime and Bond light cured adhesive to the pool helps to thin the mounting medium and allows for better adhesion of the block to the slide in larger specimens.
Sectioning of Specimens

In our laboratory, the mounted block is cut/ground to 120 microns of section thickness on a Buehler Petro-Thin. The section is ground on 1200 grit emery paper and then polished to a combined 0.1 diamond and 0.5 alumina slurry on a Buehler Ecomet II and Vibromet II respectively, until reaching a 100 μm ± 5 μm section thickness. During prolonged grinding and polishing procedures, ethylene glycol is used in place of water. Sections are marked for orientation by adhering a 100 mesh transmission electron microscopy (TEM) locator grid adjacent to the upper left corner of the section.

Following polishing, the specimen can be coverslipped (preferably with ethylene glycol rather than water, as the PMMA is hydrophobic) for imaging in transmitted or polarized light. Once LM imaging is complete, the specimen is carbon coated to make it electrically conductive for BSE-SEM analysis. (One may return to some level of LM imaging with the carbon coating intact, or the coating can be removed with an ethanol swab or by brief polishing.) The method presented in this paper allows for the sequential imaging of specimens by both LM and SEM modes.

Results

This technique has been applied to nondecalcified bone and tooth material in preparation for imaging using transmitted LM, polarized light microscopy, and BSE microscopy. It has been applied equally well in applications to large specimens (i.e., human mid-shaft femur) and small specimens (i.e., long bones of rat). Although our specimen preparation technique has been largely successful, there are several issues related to the preparation protocol that need to be discussed. In the Materials and Methods section of this paper we presented a variety of alternative experimental specimen processing techniques. These variations on the basic protocol were attempts at improving the quality of the final section.

One of the unique aspects of our preparation protocol is the use of a 50/50 mixture of heptane-isopropanol for specimen dehydrating and defatting. It was used as an alternative to the more traditional chloroform-methanol reflux (i.e., Boyd 1984) as chloroform and other polyhalogenated hydrocarbons are difficult to remove once absorbed into the bone. They may act as polymerization chain terminators via radical transfer, thereby interfering with embedding in styrene and methacrylate monomers (Odian 1981: 235). The heptane and isopropanol mixture efficiently removes lipids without these problems.

We have attempted to use a more thorough dehydrating protocol than is normally used in mineralized tissue preparation for a specific reason. Exposure to the SEM vacuum leads to cracking of the bone imposed by artifactual dehydration shrinkage. By using an intensive and careful dehydration and embedding protocol (Roschger et al. 1993), as well as by limiting exposure to water during processing, we hoped to minimize such artifact in our specimens.

As an adjunct to our traditional extended chemical dehydration procedure, we experimented with two laboratory appliances allowing vacuum dehydration (the SpeedVac, hoping that this would be less aggressive than exposure to the SEM vacuum, and CPD). We found that dehydration by these methods was very thorough, resulting in excellent infiltration of the bone with PMMA. Unfortunately, as Speed-Vac dehydration resulted in surface tensions generated on all bone surfaces with the withdrawal of water, specimens generated large macrocracks that were visible by eye.

Although this dehydrating instrumentation reduced specimen microcracking in comparison with chemical methods, none of the methods we have reported here have eliminated these artifacts completely. Other researchers have suggested that dehydration methods can never completely remove structural water in cells and matrix which leave the sample when exposed to high vacuum (Boyd and Jones 1996).

The other specimen preparation difficulty commanding our attention has been related to the retraction of dental adhesive resins. These resins tend to shrink away from the specimen as they cure. This shrinkage effect has been well documented in these adhesives (Tarle et al. 1998, Versluis et al. 1998), and its minimization has been the key to improving the bonding strength of these adhesives. In relation to our polarized LM imaging, this shrinkage causes artifactual brightness (a “halo” effect) at the interface between resin and specimen, rendering automated and quantitative imaging somewhat problematic.

We have attempted to minimize this halo effect by a number of procedures. The Prime and Bond 2.1 and Probond adhesive systems by Dentsply were chosen because of our observation that shrinkage of these resins was noticeably reduced, relative to other resins (i.e., All Bond 2, Bisco; ScotchBond, 3M, St. Paul, Minn.). For this reason we found that problems encountered in polarized light imaging of specimens mounted with more highly retracted resins were minimized.

In an attempt to improve our embedding so that the PMMA does not also retract from the specimen, we attempted to introduce a substance (see step II in our Materials and Methods: Embedding) that would prepare the bone surface to enhance its affinity to PMMA. We expected that the styrene sulfonate would form ionic bonds with the proteins, displace some phosphate on the bone surface, and react with bound calcium. This treatment would leave the specimens more hydrophobic, thereby enhancing their affinity for the PMMA. While future and very high resolution studies of embedded mineralized tissues may find some utility for this embedding variation, we did not observe the hoped-for increased affinity for the bone surface sufficient to prevent retraction of the PMMA from the bone.

Discussion

Despite artifactual cracking following exposure to SEM vacuum [which occurs even in unmounted and embedded
bone blocks (Boyd and Jones 1996, Skedros et al. 1997)), the sections produced by this embedding/mounting technique are compatible with both LM and EM modes. The mounting medium is soft enough to allow for efficient grinding and polishing, yet hard enough to be tolerant of the SEM beam. Light-cured dental adhesives have been successfully used as an SEM mounting medium in previous studies (Kristof 1997) and are optically clear for use in LM.

A distinct advantage to this method is its time savings. The use of light-cured dental adhesives allows a specimen to be processed in a very short period of time—less than 1 h of specimen handling time, save for the embedding and polishing procedures. Other condensation-cured mounting resins that rely on solvent evaporation (i.e., epoxy resins) take several days to cure completely. Visible light-cured dental adhesives cure within seconds, thus they are even simpler to use than ultraviolet-cured resins (which may take several hours to cure) that have sometimes been used for histologic procedures (Silverman 1986).

Using the same prepared section for both LM and SEM imaging allows same field-of-view images to be analyzed for their degree of relationship between such features of the specimen that are not observed by LM or SEM alone (Goldman et al. 1998, in preparation). For instance, in our research we have examined same field-of-view images acquired from circularly polarized LM and BSE microscopy in order to examine the relationship between collagen fiber orientation and mineralization of bone microstructure (Goldman et al. 1998, in preparation). We have also used BSE-SEM and fluorescence microscopy comparisons in work related to lamellar periodicity in rats (Bromage et al. 1998).

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