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Evaluating Demineralization and Mechanical Properties of Human Dentin With AFM

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1. Introduction

Atomic force microscopy (AFM) is a valuable technique for the study of demineralization and the effects of other solutions and environments on the structure of human dentin because high-resolution studies of changes in structure and dimensions are possible in nearly any environment over time ***(1)***. Because dentin forms the bulk of the tooth and is subject to a variety of alterations as a result of disease, age, and treatment ***(1)***, such studies are useful for understanding the basic structure–property relations of dentin and for evaluation of various conservative and restorative dental treatments. Initial studies of demineralization and dehydration of dentin were reported in 1993 ***(2,3)*** in which relative changes in dimensions were used to evaluate these processes. It soon became apparent that a stable reference layer on the surface or embedded in the specimen would be most desirable for evaluation of time-dependent changes and to take advantage of the high-resolution capabilities of the AFM. In 1995, the use of paint-on varnish or photoresist ***(4)*** and evaporated gold layers ***(5)*** were developed that allowed evaluation of demineralization, either continuously or sequentially, for a variety of demineralizing agents. An additional technique of interest is the use of AFM-based nanoindention for measurements of site-specific hardness and elastic modulus of dentin. Such measurements can frequently be performed as a portion of the same study in which demineralization experiments are conducted. The development of indentation-based mechanical properties measurements stems from the work of Doerner and Nix ***(6)*** and can be performed with a variety of equipment types. We have been most interested in modified AFM methods because the AFM offers the possibility of both site-specific indentation and high-resolu-

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tion imaging in solution or on dry tissue, whereas many other methods allow only the study of dry tissue. Our initial work with AFM-based nanoindentation used a modified and stiffer cantilever and diamond tip that allowed hardness measurements to be made on either peritubular or intertubular dentin ***(7,8)***. However, modified transducer heads are now available that can measure load and displacement independently and provide site-specific load displacement curves and imaging in many environments. They offer substantial improvement and allow the determination of both hardness and indentation modulus (or reduced elastic modulus). Thus, we use this method in all current measurements of hardness and elastic modulus for dentin ***(9–13)***.

1.2. Continuous and Sequential Longitudinal Studies and Reference Layers

Continuous scanning is conducted by obtaining images continuously during demineralization in the wet cell of the AFM. Thus, each pixel of a line and each line of a scan represent different exposures and offer a continuous record of the demineralization process. In sequential scanning, a baseline image in water is obtained, and then the sample is removed, treated for an appropriate exposure time, washed, and reimaged in the AFM wet cell. This method has the advantage that start and stop times can be more easily controlled and each image represents a single exposure time so that changes can be measured on all the structures in an image.

Neither the varnish nor the evaporated gold procedures were ideal for establishing a stable reference area. For the varnish method, the thickness can be difficult to control, and if the thickness is too great, it makes a poor reference. In addition, the edge is usually rounded so that an abrupt step is not apparent and it is not always clear when the top surface is flat. Having a flat reference is useful when image processing, such as plane fitting, is used. Finally, the edge where varnish and dentin meet is the area that is used for measurement. This edge is often difficult to demineralize uniformly so that a typical image may have a rounded reference layer and a zone of uneven etching in the image field of interest, as shown in **Fig. 1**.

Thin evaporated gold layers (approx 10 nm) overcame many of these problems ***(5)*** and allowed measurement between the gold layer and intertubular and peritubular dentin structures. However, there were two remaining problems with this method. Firstly, the dentin had to be subjected to a high vacuum treatment during gold evaporation. This may be undesirable in a naturally hydrated tissue and introduces microstrains ***(3)***. Second, demineralization for long periods in dilute solution or with more concentrated acids resulted in etching under the edge of the gold layer. When this occurs, the gold area is no longer a stable reference and any measurements are subject to greatly increased error.

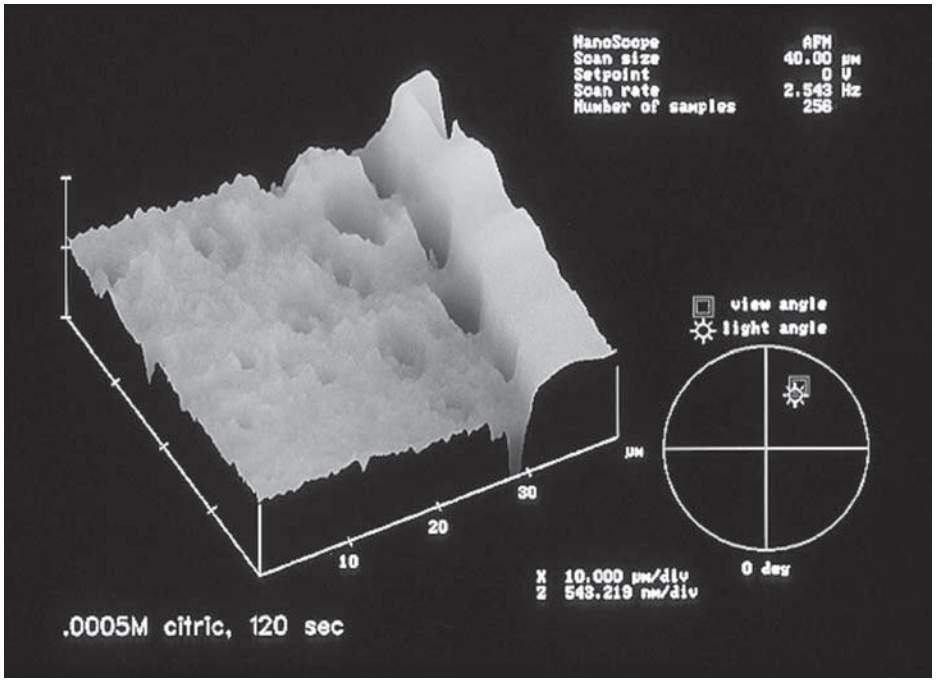

Fig. 1. Image of uneven etching that can be caused by a rounded edge of varnish, or the inability to apply the demineralizing agent uniformly at the edge of the reference layer. The etching time was 120 s with dilute citric acid. The reference area is in the upper right. A deep trough is adjacent to the layer and the uneven etching decreases with distance from the reference area, so that it is not possible to tell which areas of the dentin are representative of the etching exposure.

Three improved methods have been developed that can be used alone or in combination to overcome many of the preceding limitations. These include the use of an embedded cyanoacrylate reference layer that has been useful for demineralization studies in many dilute solutions as well as dehydration and rehydration studies ***(14)*** and an embedded glass reference layer that has been developed for studies in which the cyanoacrylate method is unstable ***(15)***. The third method is to use a tape mask to protect portions of the sample from the solution, allowing the protected portion to serve as the reference area. This has been useful for studies of single exposures to concentrated solutions and the effect of drying and rehydration ***(16,17)***. We recently have used the combination of a mask and embedded layer to evaluate the combined effects of demineralization and deproteinization of dentin ***(12)***.

1.3. Testing the Reference Layer Stability

When evaluating the effect of any new solution, the first step is to determine the stability of the reference layer. For example, cyanoacrylate is evaluated by bonding glass slides together with the cyanoacrylate, embedding in acrylic, followed by sequential metallographic polishing of the glass–cyanoacrylate–glass interface through a 0.05-μm alumina slurry. The sample is then imaged in the AFM, exposed to the solution of interest for appropriate periods, and then reimaged to determine any changes in height of the cyanoacrylate relative to the glass. For many solutions that we have studied, the cyanoacrylate has proven to be stable for exposure periods of at least 30 min. Preparing the cyanoacrylate embedded layer in dentin samples will be described in **Subheading 3.** We have found that for some solutions, such as ethanol and acetone, the cyanoacrylate is not stable, and this has led to the development of a glass reference layer method that requires additional steps for fabrication as described elsewhere ***(15)***. For dentin samples that have been etched extensively and then undergo dehydration, the interface between reference layer and dentin is damaged and often fails because of the drying stresses. To overcome this problem the extent of the etching must be limited ***(14)*** so that the interface withstands these stresses, or a masking technique can be used, in which part of the sample is protected from the acid and the unetched portion serves as a reference area. This method has been used in several studies ***(12,16–18)*** and only requires the identification of a tape that can be applied to the dentin and removed without leaving a residue. One such tape is Scotch Mounting Tape (3M, Minneapolis, MN). It also should be noted that the methods described here could be used for other calcified tissues. **Fig. 2** shows an example of enamel etched to reveal the enamel prism structure with an embedded cyanoacrylate reference layer.

2. Materials

1. Obtain teeth from human subjects following protocols approved by Institutional Review Boards and with informed consent.
2. Store whole teeth in filtered and purified water or Hanks' balanced salt solution (HBBS) at 4°C.
3. Teeth are potentially infectious and therefore should be disinfected or sterilized. Normally, we sterilize using low dose gamma-radiation ***(19)***.
4. Tooth sectioning is conducted using a low-speed water-cooled diamond saw (Isomet Low Speed Saw, Beuhler, Ltd., Lake Bluff, IL).
5. Store cut sections in HBBS at 4°C.
6. Sequential grinding is done using sand paper from 240, 320, 600, and 1200 grit (Buehler Ltd.).

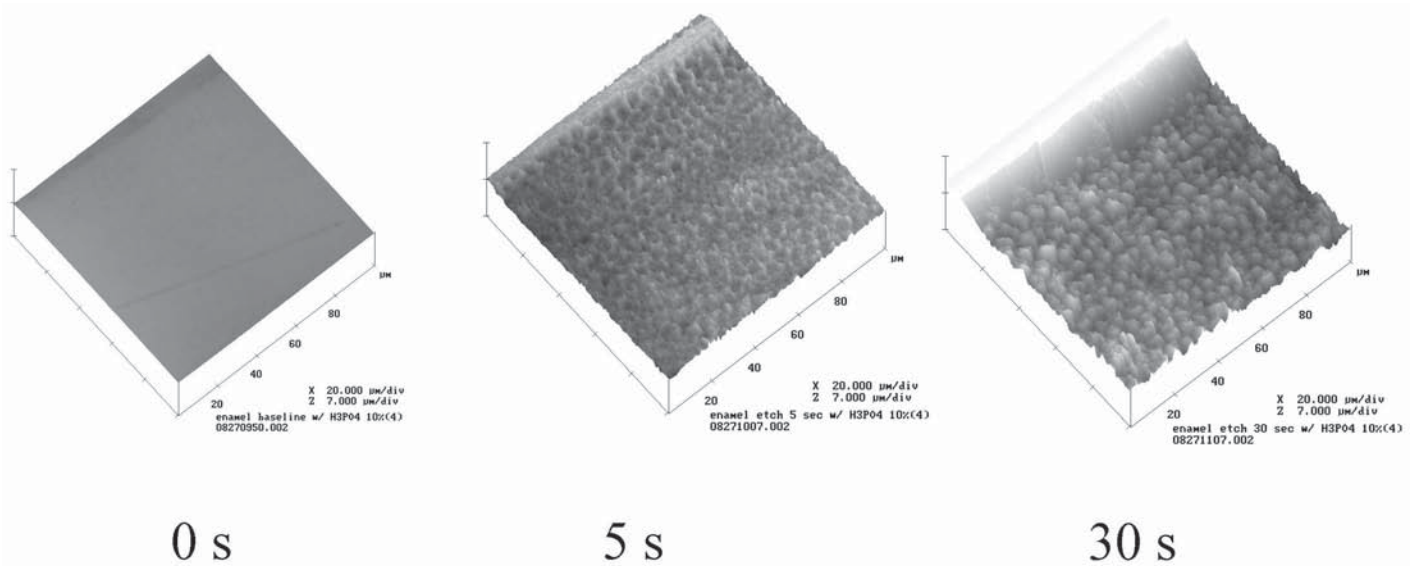

Fig. 2. Enamel etching sequence using the cyanoacrylate reference-layer method. Images for etching exposure to 10% phosphoric acid are shown at 0, 5, and 30 s. Also note that the etching pattern changed from one type to another during this treatment. The reference layer along the upper left of each image.

7. Final polishing is performed using aqueous suspensions of alumina powder or diamond (Buehler Ltd.). A fine polish will leave the structure with no detectable smear layer. Ultrasonic clean for 10–15 s between polishing steps.
8. Embedded reference layer is made from ethyl α-cyanoacrylate (MDS Adhesive QX-4, MDS Products, Anaheim, CA).
9. Solutions are usually prepared from reagent grade chemicals except for evaluations of commercial demineralization agents.
10. AFM imaging performed with a Nanoscope III (Digital Instruments, Santa Barbara, CA).
11. Nanomechanical properties (hardness and reduced elastic modulus) are ascertained with a Triboscope indentor system (Hysitron Inc., Minneapolis, MN).

3. Methods

3.1. Basic AFM Mode

AFM methods are well established. For studies of dentin recession and demineralization, we normally use the contact mode and standard S_3N_4 tips. Tapping mode also can be used and allows the tip force to be reduced. When coupled with high-aspect ratio Si tips with a very small tip diameter, it is most useful for high-resolution studies of particular features of the dentin. However, most steps of the demineralization studies do not require the high-resolution tips, which are expensive and fragile, and, therefore, the less-expensive standard tips and contact mode are used for most studies. Because dentin is a naturally moist hydrated biological composite, nearly all imaging is done in a wet cell filled with purified water at ambient temperature.

3.2. Dentin Disks With Embedded Reference Layer

1. An extracted human tooth is usually the sample of interest for studies of dentin. Because the tooth is a potential source of infection, after extraction we store the teeth in vials in distilled water or HBBS and sterilize with a low dose of gamma radiation ***(19)***. The tooth in its solution is then stored at 4°C until prepared.
2. The tooth is removed from its storage vial, cleaned of any soft tissue, and mounted on a wooden tongue depressor with hot glue. Portions of the tooth that are not of interest are usually cut off (e.g., pulp chamber and roots), and then the crown is sectioned longitudinally (sagittally) into two halves, as shown in **Fig. 3A**, using a water-cooled diamond cut-off saw (Buehler, Ltd Isomet Low Speed Saw, Lake Bluff, IL).
3. The cut surfaces (**Fig. 3B**) are then carefully polished through successive grits of sand paper (240–1200 grit) under flowing water, followed by aqueous suspensions of alumina in successive steps (1 and 0.3 μm) and ending with 0.05-μm alumina. Between steps, the sample is ultrasonically cleaned in distilled water for 10–15 s to remove remnants of the abrasives. Aqueous suspensions of diamond are another good alternative.

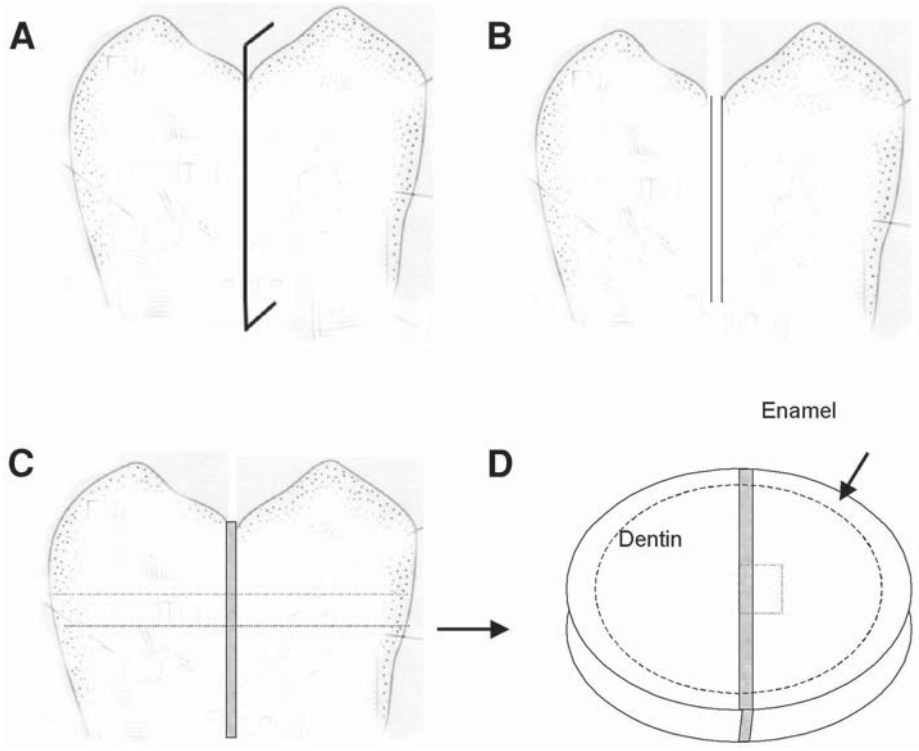

Fig. 3. Schematic diagram for construction of the cyanoacrylate reference-layer method. **(A)** Sample is sagittally sectioned; **(B)** two halves are polished; **(C)** two halves are bonded together and a disk is cut parallel to the occlusal surface; **(D)** the disk has an enamel periphery and inner dentin region with a thin layer (not drawn to scale) of cyanoacrylate that is used for the reference layer and area for study (small square).

4. The two polished halves are then blotted dry and bonded back together (**Fig. 3C**) with ethyl α-cyanoacrylate (MDS Adhesive QX-4, MDS Products, Anaheim, CA) using medium finger pressure (**Note 3**). The viscosity of the cyanoacrylate can be adjusted by air drying to increase viscosity or thinned by mixing with acetone.
5. Sections for AFM study then can be obtained by sectioning the reassembled tooth parallel to the occlusal surface using the same water-cooled diamond cut-off wheel (**Fig 3D**). Because the dentin structure varies with intratooth location ***(1)***, it is often of interest to prepare disks representative of either superficial dentin (close to the enamel) or deep dentin (close to the pulp chamber). Usually one or more disks of 1- to 1.5-mm thickness are obtained from a single tooth by repeated sectioning parallel to the occlusal surface (**Fig. 3D**).

6. After removal (**Fig. 3D**), the disk has a periphery of enamel surrounding the dentin and a thin cyanoacrylate bonded layer of about 10 μm in thickness through its center. This layer serves as the height reference layer in studies of demineralization and solution effects on the dentin.
7. The surface to be studied (either top or bottom) is then prepared using the same polishing steps, with ultrasonic cleaning between steps, which were described previously for the cut surfaces that were bonded together to reassemble the tooth. Various areas to one side or the other of the reference layer can be studied, first at baseline in water, and then after exposure to the selected treatment solution for various times. (*See* **Note 1**.)

3.3. Solutions

Typically, a dilute acid solution is prepared from reagent grade chemicals. Solutions with a pH of about 2 work well with this method and allow sequential measurements of the changes in height, relative to the reference layer, to be made at different locations in each field of view. However, nearly any solution of interest, including commercial etchants, can be used. Concentrated solutions etch the structure more rapidly and therefore the structural changes may be difficult to follow, which is the reason that dilute solutions are preferred. For example, in dilute solutions at pH 2–3, the changes in peritubular dentin can be sequentially measured after 5-s exposure steps for 20- to 60-s cumulative exposure time. After this, it is difficult to see the peritubular dentin because it etches more quickly and recedes below the level of the surrounding intertubular dentin. In contrast, a single 5-s exposure with a more concentrated acid, for example, 10% citric acid or 35% phosphoric acid, typical of those used for bonding procedures, will etch the peritubular dentin below the surface and will not allow contact with the AFM tip, so measurements cannot be made. Measurements of the changes in the intertubular dentin can be made with dilute or concentrated solutions.

3.4. Imaging and Demineralization Treatments

1. Usually initial images (baseline, *see* **Fig. 4A**) are taken by placing the polished dentin disk in the wet cell of the AFM and taking three to four images along the reference layer with dimensions of 20 μm × 20 μm to 50 μm × 50 μm (*see* **Note 2**).
2. Height differences between the reference layer and various locations in the dentin then can be determined, using the section analysis software program (**Fig. 4**). The image is processed using plane fit analysis procedures on the reference layer that was highly polished and is assumed to be flat. Site-to-site measurements are made of height differences between the reference layer and various locations on the peritubular dentin surrounding the dentin tubules and the intertubular dentin areas between the tubule units (**Fig. 4B**).

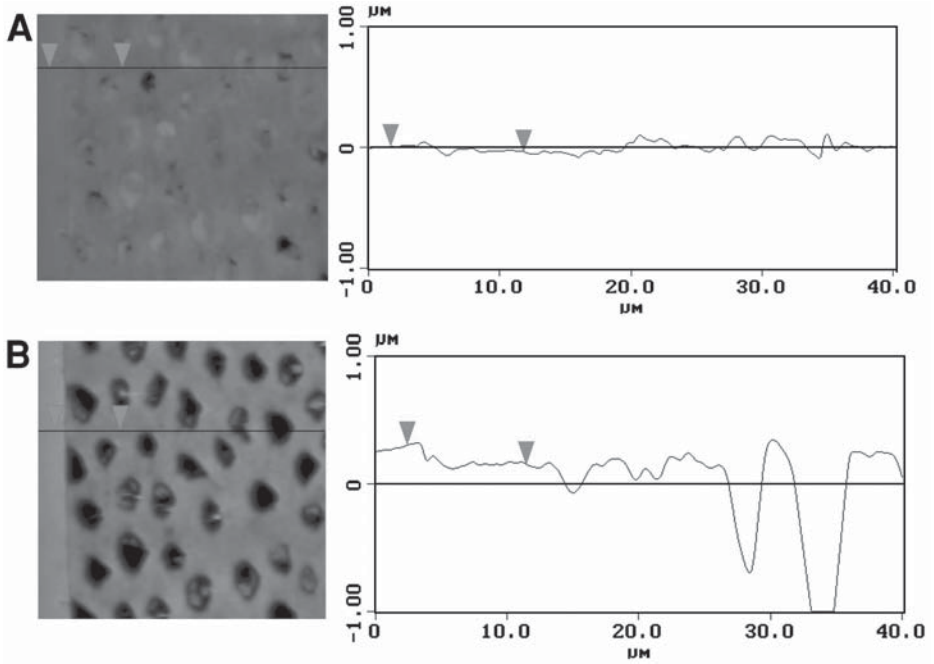

Fig. 4. Example of baseline and etched samples of dentin demineralized with citric acid These images are from the same area and the reference layer is at the left. **(A)** Baseline image in water of a 40 × 40 µm field of view. Many tubules are filled because this image was taken from transparent dentin that has mineral deposits inside tubule lumens. At the right side of the image is a section analysis showing height differences between selected points on the reference layer and the intertubular dentin (difference was 41 nm). **(B)** Same area after 30 s of etching showing enlarged tubule lumens and recession of the intertubular dentin as shown by the shading differences and the section analysis at the right. The height difference between selected points was 153 nm.

3. Normally, three to five measurements each for peritubular dentin and intertubular dentin are made per image. If there are other features of interest, such as intratubule mineral, these can be measured in the same way ***(20,21)***.
4. After baseline imaging, the disk is removed from the AFM and the desired solution is applied for a selected time. Application of the solution can be done in a number of ways, including immersion or application with a small sponge that is commonly provided in bonding kits (Sun Medical Co., Ltd, Moriyama, Japan).
5. After uniform application of the solution for the desired time the sample is thoroughly washed in purified water and placed back in the AFM wet cell and each of the previously imaged areas is reimaged.

6. Each image is plane fit, and measurements are made between the reference layer and the previously selected points (**Fig. 4B**). For dilute solutions, we usually use 5-s exposure increments for times up to 30- to 60-s cumulative exposure. The peritubular dentin is etched rapidly during this time period and gradually recedes below the surrounding intertubular dentin, leaving enlarged tubule lumens (**Fig. 4B**). Exposure increments are continued in steps of 10 s to a minute or more to follow continued changes in the dentin structure and the recession characteristics of the intertubular dentin. Normally we perform this procedure for cumulative periods of 1800 s, although shorter and much longer times have been used, depending on the aims of the study. The intertubular dentin also recedes initially at a high rate, but more slowly than the peritubular dentin. The intertubular recession generally slows, and after some time, appears to change very little with additional etching. Thus, when recession is plotted vs time for the intertubular dentin the surface recession appears to slow or reach a plateau. This is attributed to replacement of the mineral in the intertubular dentin with water that compensates for the differences in volume. However, if the dentin is dehydrated at any point during the procedure, the partially demineralized dentin will rapidly collapse ***(14,16,17)***. Thus, any dehydration will lead to errors in the measurements of surface recession and should be avoided. This is an additional motivation to make all measurements in a wet cell and under water.
   It should be noted that brief drying can be reversed rapidly, and therefore, inadvertent drying during handling will be overcome by re-immersion in the wet cell ***(14,16,17)***.

3.5. Sequential Surface Recession, Etching Rates, and Etching Characteristic Curves

For a selected field of view in a baseline image the point-to-point differences in height between the reference layer and each of the selected peritubular dentin locations and intertubular dentin areas give the initial surface height. Ideally, if all areas are perfectly polished, the differences would be zero, but in practice there is usually a small difference because the peritubular dentin, intertubular dentin, and reference layer; all have different hardness and different response to polishing. Our experience has been that peritubular dentin protrudes slightly above the intertubular dentin and the reference layer is usually at the same or a slightly higher level as well (**Fig. 4A**). The same locations are measured for each exposure interval using plane-fit processed images, based on the assumption that the reference layer is flat. Clearly, the more accurately the same points can be selected in sequential images, the more accurate will be the measurements of recession. **Figure 4B** illustrates a 30-s etch in dilute citric acid for the area shown in **Fig. 4A** and illustrates the measurements of height difference between the peritubular dentin and the reference layer, and between the intertubular dentin and the reference layer using the section analysis proce-

dure. After all the height differences from all the images are collected and corrected for any difference in height between the selected points and the reference layer at baseline, recession-vs-time curves can be constructed for peritubular dentin and intertubular dentin. As noted earlier, the recession curve for peritubular dentin can be measured only for a short time because of the rapid recession below the intertubular dentin that does not allow accurate contact with the pyramid-shape of the AFM tip. However, with data at exposure times between 0 and 20–30 s, the recession-vs-time curve can be used to estimate the etching rate of the peritubular dentin for the given solution. A similar procedure can be used for intertubular or other dentin structural components. However, the slowing of recession and the appearance of an apparent plateau in the recession for long periods makes it difficult to characterize the intertubular dentin behavior from a single short-term rate. We usually estimate the level and/or time at which the plateau occurs to characterize the intertubular dentin etching behavior (*see* **Fig. 5**). If this procedure is adopted, the features of the plateau are used rather than an estimated etching rate to describe the intertubular dentin. This seems appropriate because the AFM tip measures the intertubular dentin surface location, which is really a demineralized collagen network after an etching exposure, rather than the location of the demineralization front. The demineralization front increases with depth of continued etching although the surface recession appears to plateau, i.e., the recession rate approaches zero while the true etching rate does not. The difference between the location of the demineralization front and the apparent plateau can be determined with other techniques such as dehydration of the specimen ***(1)***, high-resolution computed tomography imaging ***(4)***, or scanning electron microscopy of fractured cross-sections.

The apparent plateau is probably not a real plateau and in fact depends on the overall depth of etching. When the location of an apparent plateau is compared with the extent of etching, samples with markedly deeper total demineralization demonstrate higher values for the level of the apparent plateau (greater recession). Thus, we speculate that the maximum value for the apparent plateau depends on total demineralization depth. This value could be determined by total demineralization of the sample and would, therefore, depend on the thickness of the original dentin disk. The experiment implied by this speculation has not as yet been conducted. If true, this means that the recession of the intertubular dentin would slowly increase with increased demineralization of the sample until a true plateau is reached, which is dependent on the thickness of the dentin disk. Despite these limitations, the apparent plateau and the time to reach this value has proven valuable in characterizing differences in demineralization response with different solutions and with different types of den-

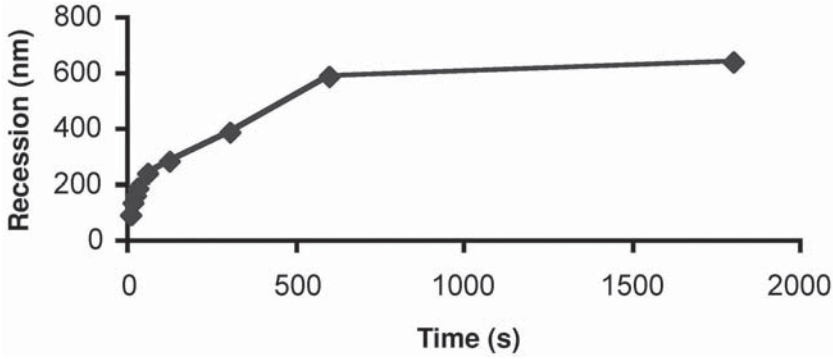

Fig. 5. Long-term decreases in height relative to the reference layer (recession) for demineralized intertubular dentin as a function of etching time in dilute citric acid for normal dentin. The recession level or time at which a plateau is observed is often useful in describing the characteristics of a particular form of intertubular dentin.

tin, such as caries-affected transparent dentin and sclerotic dentin ***(20,21)***. However, this also raises the question of how best to characterize the intertubular recession curve, define the plateau, and compare recession curves for intertubular dentin treated in different experiments. We have used different approaches to this problem. The apparent level of the plateau can be defined by a fixed criterion, for example, the point at which there is a change in dimension of less than some amount, from then until the conclusion of the experiment. With this level defined, statistical comparisons can be made to compare either the time or level of the plateau for different experimental conditions ***(20,21)***.

3.6. Other Dimensional Change Measurements

The effect of dehydration leading to the collapse of the demineralized dentin matrix (collagen) was previously mentioned and is of considerable interest from the standpoint of dentin bonding procedures, clinical treatments, and understanding the role of moisture in the dentin structure. In some experiments we have evaluated the effects of dehydration and rehydration using both the preceding reference layer method ***(14)*** and other masking methods ***(16,17)***. A difficulty arises in using the cyanoacrylate method because the etching procedure weakens the interface between the reference layer material and the dentin. On dehydration, the drying stresses often disrupt the layer and therefore do not allow measurements to be made. The weakening of the interface, as might be expected, increases with the extent of demineralization, so that for samples with shallow demineralization, the effect of dehydration and its reversal by rehydration can be followed. However, our experience has been that with

deeper (prolonged or higher concentration solutions) the interface is largely destroyed and other methods need to be used ***(16)***.

3.7. AFM-Based Nanoindentation for Hardness and Elasticity Measurements of Dentin

The same sample preparation methods can be used to prepare samples for mechanical properties measurements using the AFM. Although initial measurements were reported using a stiffer cantilever and diamond tip ***(7,8)***, improved equipment allowing recording of load-displacement curves now permits both hardness and elastic modulus determinations to be made. For these kinds of measurements the standard AFM head from the Nanoscope III is replaced with a Triboscope indenter system (**ref. *9***). In this configuration, the standard AFM head is replaced by a capacitive sensor. The sensor consists of two fixed outer drive plates that are driven by AC signals 180° out of phase relative to each other. Because of the small spacing between the two plates, the electric field changes linearly from one to the other. Therefore, the electric field potential is highest at the drive plates and zero at the center between the two plates. The center, or pickup, electrode is suspended in a manner so that it moves up and down in the region between the two drive plates. The pickup electrode assumes the electric potential of the space between the two drive plates. This results in a bipolar output signal that is equal in magnitude to the input signal at the maximum deflection, and zero at the center position. The synchronous detector converts the phase and amplitude information from the sensor output into a bipolar DC output signal. The output signal is actually a reading of the pickup electrode position. In the imaging mode, this signal is used as a feedback to the piezoceramic tube for constant force contact imaging. In the indentation mode, the feedback is cut off and a voltage ramp is applied to the lower drive plate. As a result, an electrostatic force is generated between the pickup electrode and the drive plate. The force can be described as follows:

$$F = k_e V^2 \tag{1}$$

where k_e is the electrostatic force constant and V is the applied voltage. The voltage ramps are formulated to produce triangular, trapezoidal, or square force loading profiles of the sample. For experiments in air, the force is applied to the sample through a diamond tip glued to a tapped polymer holder attached to the pickup electrode by a small screw. In liquid, the tip is glued to a tungsten rod with a large aspect ratio, which in turn is attached to the polymer holder. In this configuration, the diamond tip and portion of the tungsten rod are immersed in the solution and as a result the meniscus force remains constant as the height of liquid changes because of vaporization. This force can be easily accounted for before contacting the sample. In the imaging mode the minimum contact

force applied to the sample is about 1 μN. In the indentation mode, loads up to 30 mN can be applied. A variety of indenter shapes are available, including Berkovich and cube corner indenter. Because image quality depends on the tip radius-of-curvature, the image quality using this system may not be as good as that using the conventional AFM cantilever. However, imaging with the indenter tip allows precise selection of the area or region to be indented, and postindent imaging allows verification of the location and sharpness of each indent. The sharp cube corner indenter displaces more than three times the volume of the Berkovich indenter at the same load.

The cube tip is particularly useful when attempts are to be made to fracture a particular feature. The hardness, H, is calculated on the basis of maximum force, F_{max}, divided by the projected contact area at maximum load, a, whereas the indention or reduced elastic modulus, E^*, is calculated from the contact stiffness, S, defined as the slope of the linear portion of the force/displacement curve during unloading near the maximum load ***(6,9)***. The stiffness, S, can be determined from a polynomial expression for the unloading curve that is obtained from a least-squares fit of the data between 20 and 95% of the maximum force, and then the derivative of the unloading curve evaluated at the peak force is used as the contact stiffness, S (**Note 3; ref. *22***):

$$H = F_{max}/\boldsymbol{a} \tag{2}$$

$$E^* = \sqrt{\frac{\pi}{\boldsymbol{a}}} \cdot \frac{S}{2} \tag{3}$$

These equations appear to be straightforward, but several steps need to be taken to obtain correct values. The projected contact area, $\boldsymbol{a}$, is usually expressed in terms of contact depth, h. For an ideal pyramidal geometry tip (e.g., Berkovich), the relationship is simply as follows:

$$\boldsymbol{a} = C_0 h^2 \tag{4}$$

For a real tip the relationship can be conveniently expressed as follows:

$$\boldsymbol{a} = C_0 h^2 + C_1 h + C_2 h^{1/2} + C_3 h^{1/4} + C_4 h^{1/8} + C_5 h^{1/16} \tag{5}$$

where C_0 for a Berkovich tip is 24.5, whereas for a cube corner tip it is 2.598. To determine the relationship between h and $\boldsymbol{a}$ for a specific tip, a series of indents at various contact depths are performed on fused silica specimens and the contact area, $\boldsymbol{a}$, is calculated using Eq. 5. Finally, a plot of the computed area as a function of contact depth is plotted and fitted to Eq. 5 to obtain the coefficients in this equation.

The Young's modulus of the probed specimen, E_s, can then be obtained from E^* and the known modulus of the diamond indenter, E_i, and the known or assumed values for Poisson's ratios, ν:

$$1/E^* = (1 - \nu_s^2)/E_s + (1 - \nu_i^2)/E_i \quad (6)$$

Indentations can be made at intervals of 1–2 μm using indents of submicroscopic size (approx 300–500 nm). The spacing is needed to avoid influence of one indent on the adjacent indent (*see* discussion in **ref. *23***). Using this approach indents can be made on individual structural components of the dentin ***(10,13)*** or across junctions, such as the dentin–enamel junction ***(11)***, as shown in **Fig. 6**.

3.8. AFM-Based Nanoindentation of Demineralized Dentin

There is considerable interest in measuring dentin or other calcified tissues as modified by demineralization or hypomineralization. For example, etching procedures are used widely for bonding dentin; dentin caries (tooth decay) introduces modifications of structure that have various levels of mineralization; and genetic anomalies, such as dentinogenesis imperfecta, result in dentin of reduced mineral level. In other calcified tissues, hypomineralized or hypermineralized tissue states are of great interest. A detailed discussion of the methods for study of such calcified tissue alterations are beyond the scope of this work, but several factors need to be mentioned. Balooch et al. ***(9)*** used a modified indenter (flat-ended cylinder) to evaluate the viscoelastic properties and low elastic modulus of fully demineralized dentin when hydrated (approx 0.1 GPa) and dried (2 GPa), whereas normal hydrated intertubular mineralized dentin has an indentation modulus of about 20 GPa. Enamel modulus values are less than 100 GPa, so that AFM-based indentation methods can be used for measurement of calcified tissues over a range of at least three orders of magnitude. However, the measurements of demineralized tissue or partially demineralized tissue become extremely complex. Sharp indenter tips cannot be used reliably when the substrate consists of a demineralized feltwork of collagen fibrils ***(9)***. When a portion of the sample is demineralized and an underlying area remains mineralized, it becomes uncertain whether the underlying mineralized portion of the sample affects the measurement. We recently have studied several of these complex substrates ***(12,24)***. It is unlikely that indentations of areas with indentation modulus values below 2 GPa can be made with conventional tips. However, the limit is difficult to establish and we currently rely on careful examination of the indent shape. Furthermore, calibration of the indentation equipment and determination of the indentation tip properties are

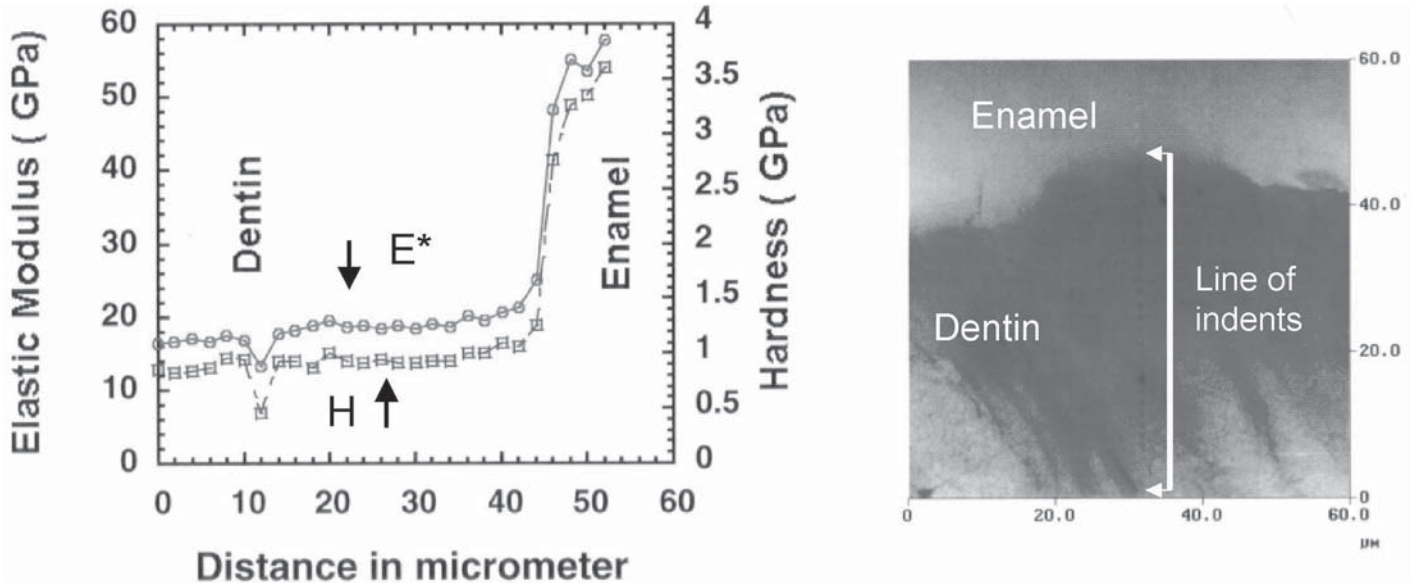

Fig. 6. Nanoindentation of enamel, dentin, and the junction between them (DEJ). The image at the right shows the line of indentations. The variations in hardness and indentation modulus as a function of position for each of the indentations are shown in the graph based on Eqs. 2 and 3.

currently carried out using quartz standards. This is an excellent procedure for mineralized tissues, but there is no adequate soft standard to insure that reliable indents can be made of the highly demineralized structures.

4. Notes

1. The cyanoacrylate viscosity is important. If the viscosity is too low, it will penetrate into the dentin and fill the tubules. This becomes evident during demineralization as the filled tubules become increasingly obvious as surrounding dentin recedes leaving them sticking up above the surface. Several cyanoacrylate gels have the desirable viscosity but were not found to be stable. Thus each product should be checked in the solution of interest. The MDS QX4 ethyl α-cyanoacrylate that we now use is too thin as dispensed and we therefore air dry a drop of the material for 2 h before bonding the polished halves of the teeth.
2. Placing fiduciary marks on the opposite side of the dentin that is to be studied can be used to facilitate identification of the same areas at each step of the demineralization experiment.
3. To obtain the real value of contact stiffness, S, we need to account for compliance (the inverse of stiffness) of the machine, C_m ***(8,10)***. The measured compliance is the sum of machine compliance C_m, and the contact compliance, C_c:

$$C_{measured} = C_m + C_c = C_m + \sqrt{\frac{\pi}{a}} / (2E^*)$$

Therefore, a linear plot of $C_{measured}$ and $1/\sqrt{a}$ for indentations made at various depths is used on a silica standard (for which C_m is well known) to determine machine compliance, which is simply the intercept of the linear plot on the compliance axis.

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