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Sample Preparation of Human Tooth Samples for Observation By Light Microscopy, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

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ABSTRACT
The human tooth sample procedure for the sample observation and analysis by light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) is the aim of the work. The experimental procedure of this sample preparation can also be used to prepare metallographic and ceramic samples.

Keywords: Sample preparation, human tooth, light microscopy, SEM, TEM.

Preparación de Muestras de Diente Humano para su Observación por Microscopía de Luz, Microscopía Electrónica de Barrido (MEB) y Microscopía Electrónica de Transmisión (MET)

RESUMEN
El objetivo del trabajo es presentar el procedimiento usado en la preparación de muestra dientes humanos para su observación y análisis por microscopía de luz, microscopía electrónica de barrido (SEM) y microscopía electrónica de transmisión (TEM). El procedimiento experimental de esta preparación de muestra también se puede utilizar para preparar muestras metalográficas y cerámicas.

Palabras claves: Preparación de muestras, diente humano, microscopía de luz, MEB, MET.

INTRODUCTION

The introduction and the rest of the text must be written 1½ spaced, in two columns, on one side of letter size paper (21.5x28) with left and right margins of 2 cm, leaving a space of 1 cm between columns. Times New Roman 10, paragraphs justified without indentation. Microscopy is the field of using light and electron microscopes to observe objects outside the naked eye resolution, and the sample preparation techniques are a crucial step in this observation. As it is well known, much of the information obtained by light and electron microscopy, both scanning (SEM) and transmission (TEM), derives in the interpretation of images after good sample preparation that allow obtaining structural and ultra-structural information [1], although most of the time the sample preparation techniques are not always completely mastered. Sample preparation techniques' importance is such that I dare to comment that more than 90 percent of the work done in microscopy is carried out in the sample preparation laboratory. The importance of handling sample preparation techniques for a researcher in microscopy is such that it provides the necessary tool to test or discard a working hypothesis [2]. The sample preparation laboratory is to microscopy as kitchen is to health in the family environment.

The subject of sample preparation covers a considerable space in microscopy congresses, where in many occasions the sample preparation symposia produce several printed volumes [3]. In fact, sample preparation has caused many researchers to receive countless awards, including the Nobel Prize. An excellent example of this is Santiago Ramón y Cajal [4], a Spanish doctor specialized in histology and pathology who received the Nobel Prize in Medicine in 1906 together with Camillo Golgi “in

recognition of his work on the structure of the nervous system". His fascination for the chemical sciences was important in developing a sample preparation method that allowed him to observe and study neurons for the first time. Another example is the Marie and Pierre Curie work [5] who developed a sample preparation process that led them to the radium and polonium discovery. In 1897 they dissolved a ton of pitchblende in acid for four years until they obtained a gram of a material that glowed in the dark. In 1903 they won the Nobel Prize for Physics together with Henri Becquerel for their work on radioactivity.

Also noteworthy is the Rosalind Frankin's work which allowed James Watson and Francis Crick to establish the double helix structure of DNA [6]. In 1962 they, along with Maurice Wilkins, received the Nobel Prize in Physiology and Medicine. More recently, Jacques Dubochet, Joachim, Frank and Richard Henderson received the 2017 Nobel Prize in Chemistry for the development of electron cryo-microscopy, which was a breakthrough in samples preparing for electron microscopy in biology sciences.

This work presents the sample preparation procedure for preparing human tooth samples for their observation by light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). This preparation process uses the metallographic method of sample preparation described by Bravman and Sinclair [7], and it has allowed obtaining excellent results in the characterization and structural analysis of human enamel and dentin. [8-10]

The Human Tooth

The main function of teeth is chewing. Teeth have three external regions: crown, neck and root. Figure 1 presents our object of study, the adult human tooth. The adult human teeth are 2 cm in size approximately. It is made up of dentin, a connective tissue that gives shape and rigidity to teeth. At the crown, dentin is covered by enamel, the strongest tissue in the human body. Enamel is responsible for protecting teeth from wear that chewing could cause. The boundary where dentin and enamel meet is known as the amelodentin junction.

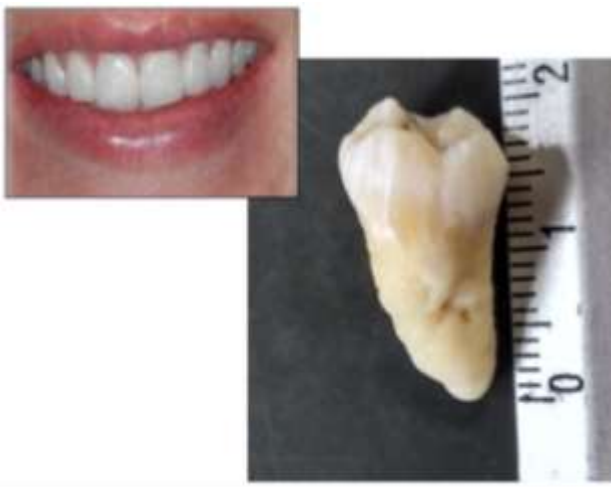

Fig. 1. The adult human tooth. The adult human tooth is approximately 2 cm in size, including the root, dentin and enamel.

Dentin is composed of 70% inorganic material, 20% organic material and 10% water. The inorganic part is a calcium phosphate named hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$), while the organic part is collagen. HAP is also the main component of bones. In dentin, two basic structures are distinguished: the mineralized matrix and, at the micrometer level, dentin tubules, holes of channels that go from the pulp chamber to the amelodentin junction. Similarly, enamel is made up of 90% inorganic material (HAP), 5% organic material and 3% water. At the micrometric level it is made up of elongated and sinuous structures named prisms. The micrometric prismatic structure is shaped in "lock"-like packs that goes from the amelodentin junction to the enamel surface. At the nanometric level, both enamel and dentin are made up of HAP crystals.

MATERIALS AND METHODS

Materials and Equipment required

The equipments used during the sample preparation and observation are:

- Diamond Disc Cutter Buehler-IsoMet 1000.
- Digital vernier Mitutoyo.
- Grinding machine Buehler-Minimet 1000.
- Ultrasonic cleaning equipment Branson Model 1510.
- Mechanical polishing machine Fishione 2000.
- Evaporator of Gold/Coal Hummer-VIA.
- Stereo microscope Zeiss.
- Light microscope Zeiss Axiovert 25.
- Scanning Electron Microscope JEOL-7800 F.
- Transmission Electron Microscope JEOL-2010F
- FIB equipment Thermo Fisher Scientific QUANTA 200–3D.

The consumables required to carry out the sample preparation are:

- Dental resin made up of monomer brand NicTone and the methyl methacrylate polymer brand Quarz.
- SiC sandpaper 400, 600, 1000 and 4000.
- Alumina Buehler MicroPOLISH of 5, 1, 0.3 and 0.05 µm.
- Micro-cloth Buehler.
- Phosphoric acid for dental use.
- Thermoplastic glue "crystal bond" or "country wax".
- Distilled water.
- Compressed air.

Experimental Procedure

Human 30-year-old permanent molar teeth from orthodontic dental treatments were used. First, a careful selection of teeth is made to avoid those with cracks and/or severe caries damage.

The general procedure, depending on whether it is to generate a sample for light microscopy, SEM or TEM observation consists of the following steps. 1) Tooth cutting, 2) Mechanical thinning, 3) Polishing to mirror finishing, 4) Preferential thinning, 5) Ionic polishing. Figure 2 shows the general procedure followed for the human tooth sample preparation. Figure 3 illustrates the procedure with images.

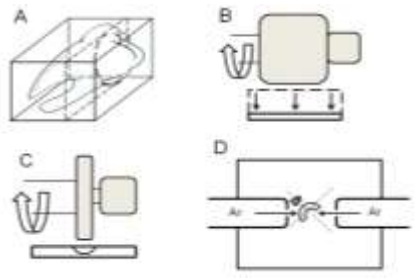

Fig. 2. General procedure for the human tooth samples preparation. A) Cutting. B) Sample's thickness reduction by mechanical grinding. C) Preferential mechanical grinding. D) Ionic grinding with argon ions.

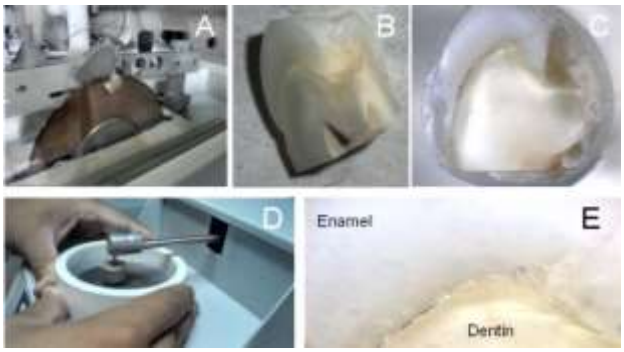

Fig. 3. Illustration of the human tooth sample preparation procedure. A) Cutting with a diamond disc. B) Human tooth after cutting. C) Immersion of the tooth into a resin to carry out the mechanical grinding. D) Thinning and polishing by mechanical grinding with SiC sandpaper. D) Human tooth sample after mirror polishing and chemical etching.

i. Step 1: Tooth cutting

The objective is to cut the human tooth with a diamond disc into 0.5mm slices containing both enamel and dentin. Teeth were cut in such a way that 3 X 3 mm samples with a thickness of 250 to 500 µm are obtained depending on whether produced samples are for TEM (thickness of 250 µm) or for SEM (thickness of 500 µm) observations. For dentin-only or enamel-only samples, the cutting can be made longitudinal or transversally.

ii. Step 2: Mechanical grinding

In this step, the thickness of the sample is reduced to less than 100 µm. This thicknesses is adequate for samples that will be observed by light microscope in the reflection mode and by SEM. The produced slices are immersed in resin to a more stable mechanical thinning. The resin is the mixing of a monomer and methyl methacrylate polymer. The resin has initially a chewy liquid consistency but soon solidifies and hardens (a small tab could be left for sample handed). Immersed in resin, the thickness of the samples is reduced until 100 µm approximately with a sequence of SiC sandpaper. Grain size in the SiC paper No. 400 is 35 µm, No. 600 is 25 µm, No. 1000 is 18 µm and No. 4000 is 5 µm. The last recess should be done with sandpaper No. 4000. This step has to be done with too much water and frequents light microscope observations to verify the flatness of the sample and if it is necessary to continue using the same sandpaper or change for the next one.

iii. Step 3: Polish to mirror finish

In this step, polish the samples to mirror finish. Set alumina of 5 µm with water on the polishing cloth. The sample is rubbed on the cloth for 5 minutes. Repeat this process with 1 µm, 0.3 µm and 0.05 µm alumina powders until reaching a scratch-free surface, always observing in the stereoscopic microscope. To clean the sample, set it in a beaker containing a mixture of 60% isopropanol and 40% acetone, and set the beaker with the sample in the ultrasound cleaner for 15 minutes. Dry the sample with compressed air.

Once the sample is polished to a mirror finish, and whether the sample is to be analyzed by light microscope or by SEM, it must be etching with phosphoric acid to reveal its structure. The acid removes the residual sludge and exposes the structure of the sample. To do this, set the sample in a Petri box and cover it entirely with phosphoric acid, allowing it to stand submerged for one minute. Finally, wash the sample with distilled water for ten minutes and dry it with compressed air.

iv. Step 4: Preferential mechanical grinding

From here, this and the next step are performed in samples to be observed by TEM. Here thinning and polishing are carried out in the central part of the sample until reach a thicknesses of less than 10 µm in the thinnest zone.

In this step, the samples continue to be mechanically thinned but now with the Fishione 2000 grinder machine. The samples are glued to the machine's holder with the thermoplastic glue "crystal bond" or "campeche wax" and preferential polishing is performed in the center of the sample with 5-mm-diameter grinding washers of the grinding equipment. The grinding will produce a concavity ("the casserole") in the center of the sample until a thickness is of less than 10 µm is obtained (which can be obtained with periodic reversals to make the grinding as homogeneous as possible on both sides).

After the concavity, polish the samples to mirror finish with alumina in the same way done in step 3. Clean the sample in a beaker with the mixture of 60% isopropanol and 40% acetone, and ultrasound cleaner. Dry the sample with compressed air.

v. Step 5: Ionic grinding

This step is only for samples to be observed by TEM, but here is also the point where the preparation of metal and ceramic samples are separated. In metals, a hole in the bottom of the casserole is easier produced by electro-polishing. In ceramics, as in the human tooth, continue as indicated in this step.

The samples are further thinned with argon ions in a Gatan machine until a small hole is produced in their center. It takes around 4 hours. The edges around the hole show the adequate thicknesses for TEM observation. Finally, the samples are covered with a 10nm thick amorphous carbon film to minimize electron beam damage and minimize the stored electron charge resulting from electron bombardment.

RESULTS

Samples for Light Microscopy

When interacting with a surface, light presents reflection, absorption and transmission phenomena depending on the physical and chemical characteristics of the sample. We used reflection of light on the dentin and enamel surfaces in mirror-polished and etching samples. The samples were observed in the Zeiss Axiovert 25 light microscope in the bright field, dark field and polarized light field observation modes with the 5x, 10x, 20x and 50x objectives. Figure 4 shows the light microscopy image in the reflection mode of the adult human tooth sample after mirror polishing and etching. Note in the structures known as "tufts" in enamel and the dentin tubules in dentin in the longitudinal direction.

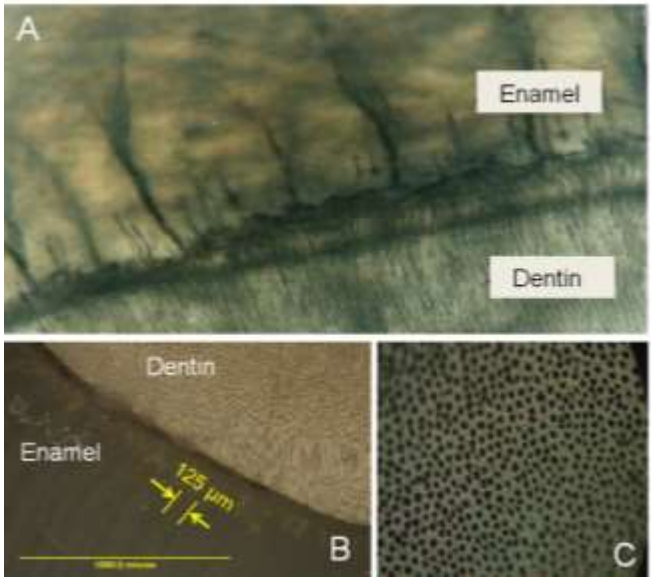

Fig. 4. Light microscopy images in the reflection mode of the adult human tooth sample after mirror polishing and etching. A) Bright field image of the amelodentin junction. B) Dark field image of the amelodentin junction. C) Dark field image of the dentin tubules.

If only the inorganic component of the tooth is going to be study, the samples can be washed with chlorine (sodium hypochlorite, NaOCl). In its commercial presentations, chlorine is in solution with 5% sodium hypochlorite and 95% water. Chlorine is a solvent for organic matter, specifically fatty acids, transforming it into soap and glycerol; it neutralizes amino-acids, forming salt and water. Furthermore, it is a disinfectant and bactericide. For chlorine treatment, prepare a solution with 60 ml of commercial chlorine and 40 ml of water in a flask and set the sample in this solution for 48 hours. When removing the sample, wash with water for several minutes.

For the transmission mode observation, the sample's thickness commented in Step 3 must be done in both sides of the sample until light passes through it. As an example of the thickness variation, Table 1 shows the thickness registered during the preparation of two samples. Note that in the case of human tooth, the sample is observed by light microscope in the transmission mode after reaching a thickness of 260 ± 0.50 µm.

Table I. Thickness values registered in two human tooth samples during grinding and polishing.

Measure Number	Thickness (±0.05 mm)	
	Sample 1	**Sample 2**
Beginning	4.32	4.40
1	2.95	3.02
2	2.53	2.57
3	2.06	2.02
4	1.54	1.52
5	1.07	1.03
6	0.51	0.55
First observation in transmission	0.26	0.23
Transmission	0.10	0.15
Last observation in transmission	0.03	0.08

Figure 5 shows the light microscopy image in the bright field transmission mode of the adult human tooth sample after thinning it to a thickness of less than 100 µm. This Figure shows the amelodentin junction and the contrast of enamel prisms and dentin tubules observed in the longitudinal direction.

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Fig. 5. Bright field light microscopy image in the transmission mode of the amelodentin junction of the adult human tooth sample after thinning it to a thickness of 100 µm approximately, mirror polishing and etched.

Samples for SEM

Samples observed by the light microscope are easily observed by the SEM microscope. Only stick the sample to SEM's barrel holders with silver paint, carbon paint or carbon double-sided tape, and evaporate a gold (or carbon depending on the type of study to be carried out) thin film of approximately 20 nm in thickness on their surface.

Figure 6 shows the secondary electron mode SEM image of the adult human tooth sample after thinning it to a thickness of approximately 100 µm, mirror polishing and chemical etching. The "fish-skin" contrast in Figure 6A is produced by the enamel prism array. Figure 6B shows the dentine-tubules holes.

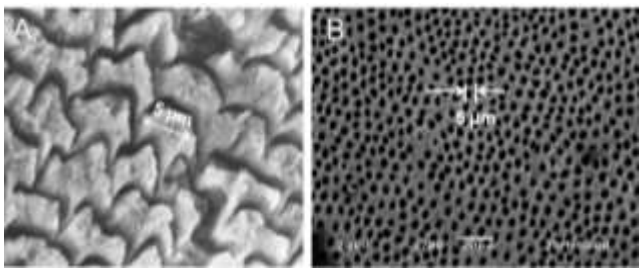

Fig. 6. Secondary electron mode SEM image of the adult human tooth sample after thinning it to a thickness of 100 µm, approximately, mirror polishing and chemical etching. A) Enamel. B) Dentin.

Figure 7 shows the backscattered electron mode SEM image of the adult human tooth sample after thinning it to a thickness of 100 µm approximately, mirror polishing and etching. Figures 7A-C shows the EDS analysis spectra from the areas indicated by the boxes in the SEM image. Note the variation of the elemental peaks corresponding in each case.

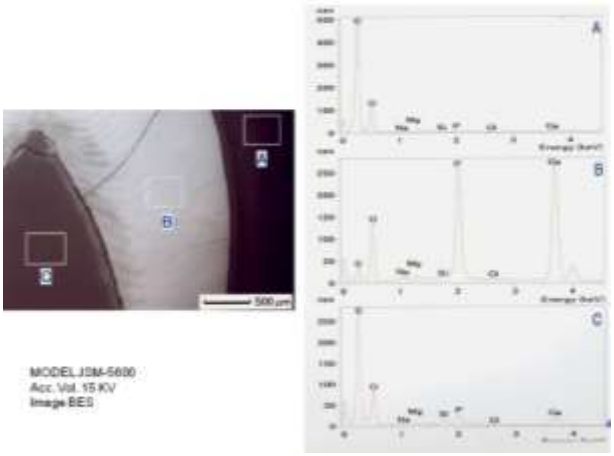

Fig. 7. SEM image in backscattered electron mode of the adult human tooth sample after thinning it to a thickness of 100 µm, approximately, mirror polishing and chemical etching. A-C) EDS spectra of the areas indicated by the boxes in the SEM image. A) Resin area where the tooth was immersed for its thinning and mirror polishing process. B) Enamel, C) Dentin.

Samples for TEM

The samples thinned with the Gatan ion machine until a small hole was produced at their center, the edges around the hole are thin enough for TEM observation. Then the sample was covered with a 10-nm-thick amorphous carbon film to minimize electron beam damage and electron charge resulting from electron bombardment during observation. Figure 8 shows the TEM image of the adult human tooth sample of the after the ion milling equipment. Figure 8A shows the bright field TEM image of enamel and Figure 8B shows the TEM image in high resolution mode (HRTEM). Figure 8C shows the HRTEM image where the atomic positions are clearly identified.

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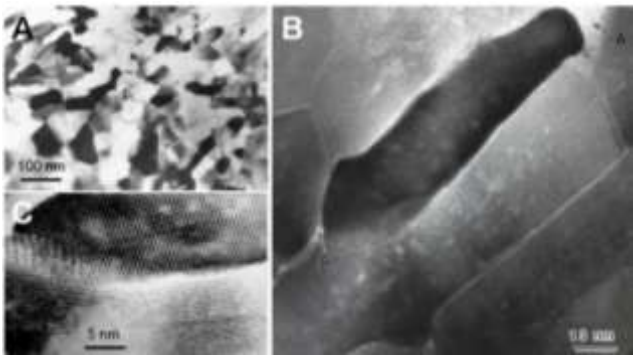

Fig. 8. TEM image of the adult human tooth sample after the ion-milling thinning and covered with an amorphous carbon thin film. A) Bright field image. B) TEM image in high resolution mode (HRTEM). C) HRTEM image.

Today, there is an equipment that produces very thin slices (of the appropriate thickness to be observed by TEM) from the samples using focused ion beams (FIB) of gallium ions. This is equipment can cost more than a TEM microscope and is built as part of an SEM microscope. Thus, SEM images are observed in situ as the FIB slices the sample. The sample to be prepared by FIB must have a pre-preparation. The sample obtained from Step 3 is the most suitable to be sliced with a FIB equipment. Once the slice is prepared, it is set on a TEM grid for observation. Here a Thermo Fisher Scientific FIB QUANTA 200–3D was used.

Figure 9A shows the secondary electron mode SEM image of a FIB slice of the adult human tooth sample. The observed steps in the slice are the different thinning cycles that were carried out until reaching the appropriate thickness for TEM observation. In this case, a thickness of 5 nm was obtained. Figure 9B shows the bright field TEM Image of the area indicated by the rectangle in Figure 9A. Here the edge of one of the enamel prisms is clearly seen. Figure 9C presents the SEM image in the backscattered electron mode from the area indicated by the rectangle in Figure 9A. Note the SEM contrast of backscattered electrons shows the HAP crystals of the enamel prism. The difference in intensity shown by these crystals is caused by the relative orientation they have with the electron beam and the backscattered electron detector.

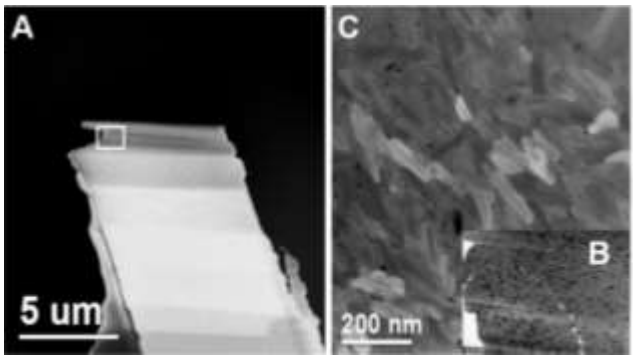

Fig. 9. A) SEM image in the secondary electron mode of the adult human tooth enamel sample the FIB slicing. B) TEM bright field image of the area indicated by the rectangle in (A). Note the edge of one of the enamel prisms. C) SEM image in the backscattered electron mode of the area indicated by the rectangle in (A).

At this point the maximum result of the human tooth sample process has been reached. Figure 10 shows the TEM images of the adult human tooth enamel sample after slice it with the FIB equipment. In this Figure, the shape and size of the HAP crystals that make up the dental enamel can be observed.

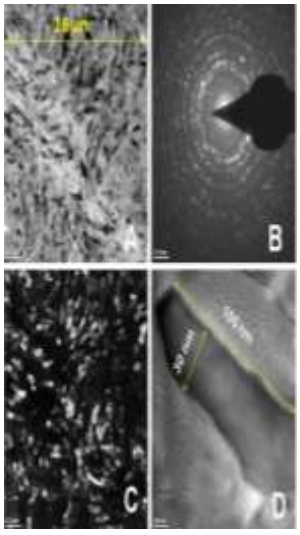

Fig. 10. TEM images of the adult human tooth enamel sample slicing with the FIB equipment. A) Bright field image. B) Select area electron diffraction pattern. C) Dark field image. D) HRTEM image of the HAP nanometric crystals of human tooth enamel.

Figure 10A shows the bright field image, Figure 10B shows the corresponding selected area electron diffraction

pattern from the area shown in Figure 10A, and Figure 10C shows the dark field image of the area shown in (A). Figure 10D shows the HRTEM image of the HAP nanometric crystals of human tooth enamel.

Figure 11 shows the case for the adult human tooth dentin after slicing with the FIB equipment. Figure 11A shows the bright field image, Figure 11B shows the corresponding selected area electron diffraction pattern, and Figure 11C shows the dark field image of the area shown in Figure 11A. Figure 11D shows the HRTEM image of the HAP nanometric crystals of human dental dentin. The analysis of some of these images have been reported everywhere [11].

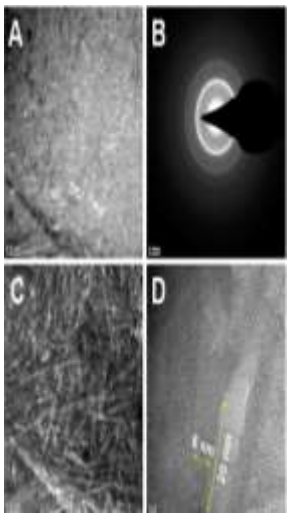

Fig. 11. TEM images of the adult human tooth dentin sample after slicing with the FIB equipment. A) Bright field image. B) Electron diffraction pattern of selected area of the zone in (A). C) Dark field image. D) HRTEM image of the HAP nanometric crystals of human dental dentin.

FINAL REMARKS

I do not want to finish this work without commenting that reaching these levels in sample preparation requires not only having the necessary materials and equipment to carry it out, but it is also necessary to satisfy certain personal characteristics. Among these is obviously having the required skill, a curious mind, knowledge of the material under study, deductibility on the behavior that the material presents, tenacity (stubbornness), a lot of imagination, and, of course, knowing how to work in a team.

An example of skill is Antoine van Leeuwenhoek whose hobby in 1632 was to polish tiny lenses and he made a microscope that magnified from 50x to 200x without distortion. He was the first to describe the light microscope images (there were not photographs but by hand drawing) of protozoans, bacteria, sperm, and red blood cells, and he published his observations in around 400 articles in The Real Society of London. Perhaps he was not the first to build a microscope, as it is say that Galileo Galilei did this invention, but Leeuwenhoek did say what to do with it [12].

To comment on the curious mentality, let us take the case of Wilhem Conrad Rontgen, who in 1895 was fascinated by the glow produced by a vacuum tube when generating an electric shock. He put the tube in a black box, darkened the room, and noticed a bright flash on a barium platinum-cyanide sheet every time he connected the tube to the power. When he set the paper in another room, the same phenomenon happened. He had discovered something invisible that was "felt" through doors and rooms, and he named it "x-ray" [12].

Having the knowledge on the subject being worked on does not need checking. An example of this point is represented by Henri Antoine Becquerel who in 1896 sought on the nature of x-rays. He studied fluorescent materials (those that glow when exposed to ultraviolet light) and their relationship to x-rays. He noted that photographic papers blurred when placed them on uranium salts and deduced that the uranium salts emitted a radiation capable of passing through opaque materials. This radiation ionized the air and was deflected by electric and magnetic fields, unlike X-rays. Initially, he named it as B-rays [12].

As an example of deductibility, let's take to Joseph John Thomson, who in 1897 was also looking for the nature of x-rays, investigated the nature of cathode rays and showed

that electric and magnetic fields could deflect them, which is not the case with x-rays. He discovered that cathode rays are made up of small particles with a negative electrical charge, which he named "electrons". Thus, the electron was the first sub-atomic particle to be discovered, and it is now known that when electrons collide with the anode of the cathode tube, x-rays are produced [12].

In the case of tenacity, no one was more tenacious than Pierre and Marie Curie to dissolve for a little over four years a ton of pitchblende. In 1903 they won the Nobel Prize for Physics together with Henri Becquerel for their study of radioactivity. Later, in 1911, Marie Curie won the Nobel Prize in Chemistry for the discovery of Radio and Polonium [12].

No one exemplifies imagination better than Ernest Rutherford. In 1908 he bombarded a 200nm thick gold foil with a beam of radioactive beams. The beam was scattered and produced a diffuse dark circle in the photograph. Conclusion: most of the atom is empty space !. He imagined electrons traveling in orbits around the nucleus, similar to a tiny solar system with the mass of the atom in its nucleus. Thus he deduces that the nucleus measures approximately one ten thousandth of the diameter of the atom and has a positive electric charge. Rutherford received the Nobel Prize in Chemistry in 1908 for work prior to his greatest contribution: the atomic model [12].

Teamwork is illustrated by itself. To reach the goal of producing a sample, it is crucial to rely on the most experienced people: the laboratory technicians. They are a cornerstone on our way to obtaining samples!

Of course, not all of us learn the sample preparation techniques at the same rate. An optimal way to know if whether or not we already have this ability is to take apart and assemble a watch (for example, a 1970's watch) or any other mechanical device that contains millimeter pieces. This exercise will immediately indicate whether or not we have the necessary requirements to prepare samples.

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MSc's:

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Carlos Eduardo Zamora Montes de Oca, 1999.
América de Lourdes Ayuso Arce, 1999.
Ruth Friedman Saavedra, 2002.
Dora Laura Torres Contreras, 2002.
Ana Guadalupe Rodríguez Hernández, 2004.
Gaby Esther Tiznado Orozco, 2004.
María Josefina Arellano Jiménez, 2004.
Margarita Ivet Gil Chavarría, 2005.
Miguel Ángel Gutiérrez Pérez, 2005.
Luis Antonio Morales Vásquez, 2005.
Rolando Harvey Santillán Velázquez, 2007.
Patricia Dettmer Mendoza, 2012.
Galois Rodríguez Álvarez, 2013.
José Antonio Rodríguez Torres, 2017.

Bachellor's:

Claudia Minerva Alcántara Rodríguez, 1993.

Ana María González Trejo, 1994.

Alfredo Madrigal Colín, 1994.

María de Jesús Gloria Hernández, 1994.

Israel Morales Sánchez, 2000.

Ana Guadalupe Rodríguez Hernández, 2001.

Margarita Ivet Gil Chavarría, 2002.

Dulce María Cruz Villaseñor, 2000

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Antonio González Vázquez, 2004.

Lizeth Redonda Pineda, 2007.

Eder Acosta Sierra, 2010.

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Javier Armando Espinosa Rodríguez, UAN, 2012.

Alexis Emmanuel Guardado Paredes, UAN, 2012.

Elías Rodríguez Ríos, UAN, 2012.

Armando Edgar Woodbury López, UAN, 2012.

Martha María Nava Ruiz, UAN, 2012.

Isabel Cristina de León Mata, UAN, 2013.

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Sandra Hernández Arellano, CECyT 2, EdoMex, 2013.

Leonardo Ociel Espinoza Zepeda, UABC, 2015.

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