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Three-sectioning method: A procedure for studying hard tissues and large pieces under light and electron microscopy

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ABSTRACT

The histological study of hard pieces such as tendons and calcified lesions and tissues is a field that has been gaining increased attention owing to the rapid development of implantable prostheses, among other factors. In these studies, serial sectioning is utilized to detect areas of interest throughout the entire piece, as it enables the application of the appropriate light and electron microscopy techniques in these areas. We propose the "three-sectioning method" that subjects the pieces to three consecutive cycles of embedding and sectioning to localize and study the areas of interest, as an efficient technique for these histological studies. The pieces were first embedded in epoxy resin and then cut into thick sections (approximately 300 μm) for the first cycle. Next, areas of interest selected on these thick sections were re-embedded in epoxy resin to be sectioned again (second sectioning) to obtain a series of semithin sections (1–3 μm). These semithin sections are usually studied using the most relevant techniques for light microscopy. Smaller areas of interest are selected to be cut into ultrathin sections (60–90 nm) for transmission electron microscopy. If necessary, the selected areas of the semithin sections can be embedded again, and then cut into new ultrathin sections. The different kinds of sections we have described here may also be studied using scanning electron microscopy. This systematic method facilitates correlative microscopy from lower to higher magnifications along with the usage of a broad variety of histological techniques including electron microscopy.

1. Introduction

The histological structure of hard tissues, such as other tissues in organisms, is the structural basis for determining normal functions. In the case of disease, it plays a key role in the pathophysiology, diagnosis, and treatment of the disease (Majno and Joris, 2004).

However, calcified or hard tissues, including bone, cartilage, tendon, etc., present important difficulties in performing histological studies. These difficulties increase when areas with different densities or hardness are involved, such as the vascular reaction to an arterial stent (Malik et al., 1998) or other medical devices (Rousselle et al., 2019). The interest in implantable prostheses is continuously expanding, and many of these prostheses are made of hard substances (Lim and Lee, 2009), such as calcium carbonate, ceramics, and metals such as titanium and its derivatives (Guillaume et al., 2020; Willbold et al., 2013). The histological study of these materials is necessary, first, in the experimental phase and later, while evaluating the results and tissue reaction produced by these devices in the surrounding tissues (Rippstein et al., 2006). In addition, it is necessary to obtain complete sections without splintering or losing a part of the sample to make, if necessary, series of sections or three-dimensional reconstructions (Bock et al., 2011).

For example, the histological study of bone pieces can be conducted using three groups of methods: previous decalcification, direct sectioning, or grinding and polishing.

The easiest method is the previous decalcification of a piece of bone, using strong acids, calcium chelators, or other methods (Kammerman et al., 1995). This method involves embedding the pieces in paraffin and obtaining sections of adequate thickness for histological studies. However, paraffin is not suitable for embedding and cutting large pieces. To improve the hardness and elasticity of paraffin-based embedding media, different types of substances, such as celloidin and polylactic acid, have been added (Gabe, 1968) but the results were not good enough and it necessitated the search for other embedding media.

Moreover, decalcification causes important artefacts that can be avoided by embedding the pieces in substances with more favorable

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physical properties than paraffin (Gabe, 1968; Savi et al., 2017).

Conversely, the direct sectioning of hard tissues is possible after embedding a small piece in a material that is hard and elastic enough to be sectioned with a glass or diamond knife. The material for this embedding could be any type of methacrylate (Wittenburg et al., 2009) or epoxy resin (Spurr, 1969). These methods for small and hard pieces enhance the possibility of using transmission and scanning electron microscopy (SEM).

The grinding and polishing methods are based on wearing away part of the material from the surface of the piece to reach a thickness of approximately 10 μm, which is appropriate for histological analysis (Sennerby et al., 1993). The grinding and polishing can be conducted on pieces previously submitted to different treatments from dried bone to plastic embedding (Gabe, 1968). These non-decalcified pieces are sectioned into thick sections (100–400 μm) using a precision saw. These thick sections are further ground and polished to reach a thickness of 5–10 μm (Donath and Breuner, 1982; Geith et al., 2014), which is useful for histological studies.

An important advance in embedding media was the utilization of acrylic resins derived from methacrylate (Caropreso et al., 2000). These resins produce, by polymerization, an embedding media harder and more elastic than paraffin, thereby facilitating the sectioning of hard pieces. To improve the physical characteristics of the embedding media, different molecules have been added to methacrylate, producing polymers such as methyl-methacrylate, ethyl-methacrylate, and glycol-methacrylate (Buijs and Dogterom, 1983; Donath and Breuner, 1982). However, these methyl-methacrylate derivatives have the drawback that the polymerization is a strongly exothermic reaction that can reach 70 °C. In addition, the accelerator is flammable, even explosive (Velde et al., 1977). The accelerator and the monomer have subsequently been modified to produce the “Technovit 9100 New”, to avoid dangerous chemical reactions. This mixture polymerizes between −2 and −20 °C and is less flammable than previous mixtures (Willbold and Witte, 2010).

An alternative to acrylic embedding media are the epoxy resins such as Epon 810 or the low viscosity epoxy resin (Spurr, 1969). Epoxy resins were introduced in the 1940s, chiefly as embedding media for electron microscopy (Blaauw et al., 1989). The low viscosity epoxy resin can penetrate more deeply, allowing the embedding of larger pieces (Rippstein et al., 2006). Good results have been achieved by (Mukhamadiyarov et al., 2016) in grafted stents embedded in epoxy resin following grinding and polishing to obtain samples which could be observed under light and scanning electron microscopes.

In the present study, we describe a method based on three sequential series of embedding and sectioning that yield thick, semithin and ultrathin sections. These sections allow the systematic study of hard tissues or prostheses with practically any type of light and electron microscopy technique, from the macroscopic to the ultrastructural level.

2. Materials and methods

2.1. Animals

In this study, 10 Wistar albino rats of both sexes and 200–250 g in weight were utilized. These animals (5 animals in each group) were part of two studies currently in progress, one on mandible bone regeneration and the other on nerve regeneration through tubular prostheses. The animals were obtained from the Animal Center at the University of Valladolid and handled in accordance with the guidelines of Directive 2016/63/EU of the European Parliament and those of our country (RD 53/2013) for Laboratory Animal Care and Experimentation. The protocols of both projects were approved by the Service of Animal Welfare and the Ethics Committee of the University of Valladolid.

2.2. Methods

The rats in the bone regeneration group underwent a critical-size (4 mm) osteotomy in the mandible (Fig. 2). The osteotomy was repaired by covering it with a piece of titanium foil with cultured autologous mesenchymal stem cells on the medial surface. The autologous mesenchymal stem cells were obtained from rat inguinal adipose tissue following the method proposed by Kuhbier et al., 2010 for human adipose tissue. Cells were cultured on square pieces (2 cm side) of 12.7 μm thick perforate titanium foil (Alfa Aesar). The culture medium was Dulbecco's minimum essential medium (Gibco) supplemented with 10% foetal-bovine serum and 1% penicillin / streptomycin (Gibco). The titanium pieces were placed onto small culture plates and maintained in an incubator (Hera Safe, Heraeus) at 37 ºC, 100% humidity, and 5% CO_2. A confluence of approximately 70% was reached before being implanted. Because the cells were cultured on an opaque support (titanium foil), the morphology and confluence of the cultured cells were ascertained with Fluopaque, an epifluorescence and a general fluorescent staining solution made of eosin Y and DAPI (Gayoso, 2012).

Nerve regeneration was studied in neurectomies of the sciatic nerve, repaired with an acellular isogenic nerve segment grafted between both ends of the neurectomy (Fig. 3). The decellularization of the nerve segments was performed using different kinds of detergents, namely, Triton X-100, sulfobetaine-16 and sulfobetaine-10 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), following the methods of Hudson et al., 2004. The decellularization eliminates the cells maintaining the internal structure of the extracellular matrix, which may serve as a scaffold for the growth of the regenerating neuronal processes. In some experiments, the decellularized nerve segment was inside a tube made of different materials such poly(ϵ-caprolactone), poly(lactic coglicolic acid), titanium foil and several types of medical tubing.

Prior to the surgery, the animals were anaesthetized using a mixture of ketamine and xylacin (0.13 ml of Imalgene 1000 and 0.02 ml of Rompun per 100 g of animal weight). After one month of survival for bone repair, and a week for nerve repair, the animals were again anaesthetized and sacrificed by transcardial perfusion. Perfusion started with a washing solution (0.1 M phosphate buffer pH 7.4) followed by the fixative solution. We used two fixation protocols, one for light microscopy and SEM studies and the other for ultrastructural studies.

For light microscopy and SEM, the fixative solution used was buffered 4% paraformaldehyde. After fixation, the pieces were left in the same fixative overnight, washed the next day in phosphate buffer, and subsequently processed by our method.

For ultrastructural studies using transmission electron microscopy (TEM), the rats were perfused with paraformaldehyde-glutaraldehyde (Palay and Chan-Palay, 1974). The next day, the pieces were postfixed in 1% osmium tetroxide for 2 h, washed in phosphate buffer, and then processed by our method. Whenever possible, all the processes involved agitation.

3. Three-sectioning method

After fixation, the pieces were processed using our method, which consists of three consecutive cycles of embedding-sectioning followed by appropriate staining. A simplified flow chart of this process is shown in Fig. 1.

3.1. First embedding-sectioning

The pieces, which were previously fixed, were washed with the same buffer three times for 15 min and dehydrated with increasing alcohol concentrations of 50%, 70%, 90%, and 100% with three changes of 15 min for each concentration. The pieces were then placed in a 1:1 mixture of absolute alcohol and propylene oxide with three changes of 15 min each, after which they were placed in pure propylene oxide, with three changes of 15 min each. After the propylene oxide

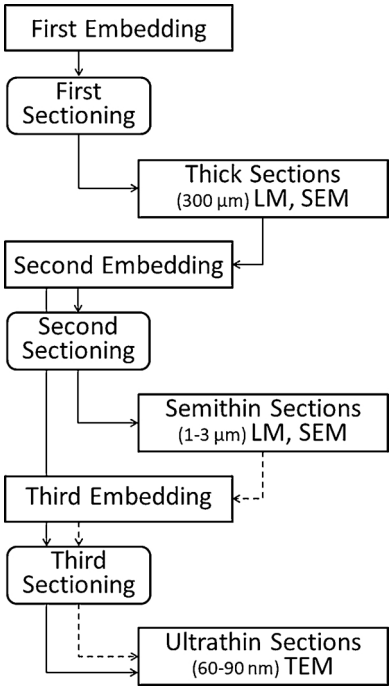

Fig. 1. Flow chart of the main steps of the three-sectioning method. The process yields three series of histological sections: thick, semithin, and ultrathin sections. Notably, the semithin sections can be embedded and sectioned again (third embedding and sectioning) to obtain ultrathin sections (dashed lines).

treatment, the pieces were placed overnight in a 1:1 mixture of propylene oxide and low viscosity epoxy resin (Spurr, 1969).

The next day, the pieces were placed in an appropriate position in a Teflon or plastic resistant non-adherent mold and covered with new pure epoxy resin. The pieces, in their molds, were placed in a vacuum bell at 200 mbar for 6 h to improve the resin penetration and extract the air bubbles that could have been formed. They became polymerized overnight at 60 °C.

After epoxy resin polymerization, the pieces were sectioned using a precision saw IsoMet ™ 4000 (Buehler) with diamond powder discs 140 µm thick. This enabled us to obtain long series of thick sections easily. These sections (approximately 300 µm thick and 2 cm long) are suitable for studies using low power light microscopy. For reflected light microscopy studies, we used a Leica M80 stereo microscope with a Leica HC100 HD camera, whereas in the case of transmitted light microscopy studies, we used an Axiophot (Zeiss) microscope with an AxioCam HRc digital camera controlled with Axiovision software. Thick sections may be stained with several histological stains such as toluidine blue (1% in 3% sodium tetraborate) for general staining, the von Kossa method (Bancroft and Gamble, 2008) and alizarin red (0.2% in deionized water) for histochemical reactions of calcium.

These thick sections can also be analyzed using different SEM techniques. We used an environmental scanning electron microscope (ESEM) (QUANTA 200 FEG) operating at low vacuum mode at pressures ranging from 0.6–0.9 Tor (80–120 Pa). This operating mode allows us to image samples while avoiding charging effects without any metallization of the piece surface. Our SEM was equipped with detectors for secondary electrons (SE), backscattered electrons (BE), and energy dispersive X-ray spectroscopy (EDXS).

3.2. Second embedding-sectioning

After morphological studies of the thick sections, areas of interest can be selected. These selected areas can be easily removed from the rest of the section with a razor blade and re-embedded in epoxy resin. After a short washing in propylene oxide, the second embedding was done by orienting the selected area in new epoxy resin in the mold and polymerizing overnight at 60 ºC.

Semithin sections (1–3 µm thick) were obtained from the second sectioning using an ultramicrotome Ultratome IV (LKB) using glass or diamond knives. These semithin sections (Fig. 1) are useful for at least three kinds of studies. The most usual, as in conventional TEM, is the selection of an area of interest under light microscopy. This area is generally localized, first in the semithin sections and then in the remaining block trimmed for the third sectioning (Fig. 1) to produce ultrathin sections. In the second use, semithin sections are suitable for high-resolution light microscopy studies, applying almost all the available histological techniques such as general staining, immunocytochemistry, and *in situ* hybridization. Semithin sections can be deplasticized, for example, with sodium ethoxide (Tóth and Czoma, 2011) to improve the performance of some stains, or heated in a citrate solution to recover the antigenicity. It is possible to apply various SEM techniques on semithin sections as already mentioned.

In the cases where an interesting structure was found only in a few semithin sections, the small pieces could be embedded again in epoxy resin (Fig. 1). This would constitute the third embedding.

3.3. Third embedding-sectioning

Embedding a semithin section requires processes similar to the other embeddings (Fig. 1), but care must be taken to ensure a flat embedding. The section was washed with propylene oxide, infiltrated in epoxy resin and deposited on a plane surface that was previously made by cutting an empty block of epoxy resin. On this surface, the section was deposited, pressed, and polymerized as before.

This phase yielded ultrathin sections similar to those obtained from the blocks of the second embedding-sectioning. The contrast was increased using uranyl acetate and lead citrate. The rest of the methodology followed is the same as that for the conventional TEM.

4. Results

One of the remarkable outcomes of our method is that the pieces to be studied can be relatively large. For example, in our study of bone regeneration we embedded (Fig. 2A) entire rat hemimandibles (3 × 2 × 1 cm). The series of sections (during the first sectioning) included the total height of the jaw, maintaining its morphology and structure. The graft area had several elements with different densities and hardness, viz., the titanium foil, the mandibular bone, cultured cells and soft tissues such as muscle, adipose and connective tissue (Fig. 2C). In view of the hardness and elasticity of the epoxy resins, it is easy to obtain a series of sections of approximately 300 µm thick without any compression that could occur when cutting areas with different hardness. We refer to these as the “thick sections” and approximately 20 of these thick sections were obtained from 1 cm of the piece (Fig. 2B and C). These series of the thick mandibular sections were usually stained with alizarin red to study the general morphology of the implant and the responses from the adjacent tissues.

After identifying an area of interest in a thick section (Fig. 2C), we cut this area off to make a second embedding and obtain semithin sections (Fig. 2D–G). In the examples we have proposed, each area of interest can yield more than 50 semithin sections. The semithin sections are suitable for a great number of histological techniques. As an example, using toluidine blue for general staining, the semithin sections showed the characteristic histological structure of cancellous bone. Cells and non-calcified tissues appeared in a blue color while the calcified bone remained mainly unstained (Fig. 2D). When the von Kossa and alizarin red methods were used (Fig. 2D and F), the calcified tissue showed the characteristic black and red colors, respectively. More structural details (Fig. 2D) could be discerned using toluidine blue than with the other histochemical methods (Fig. 2E and F).

Our method offers the possibility of observing the histological

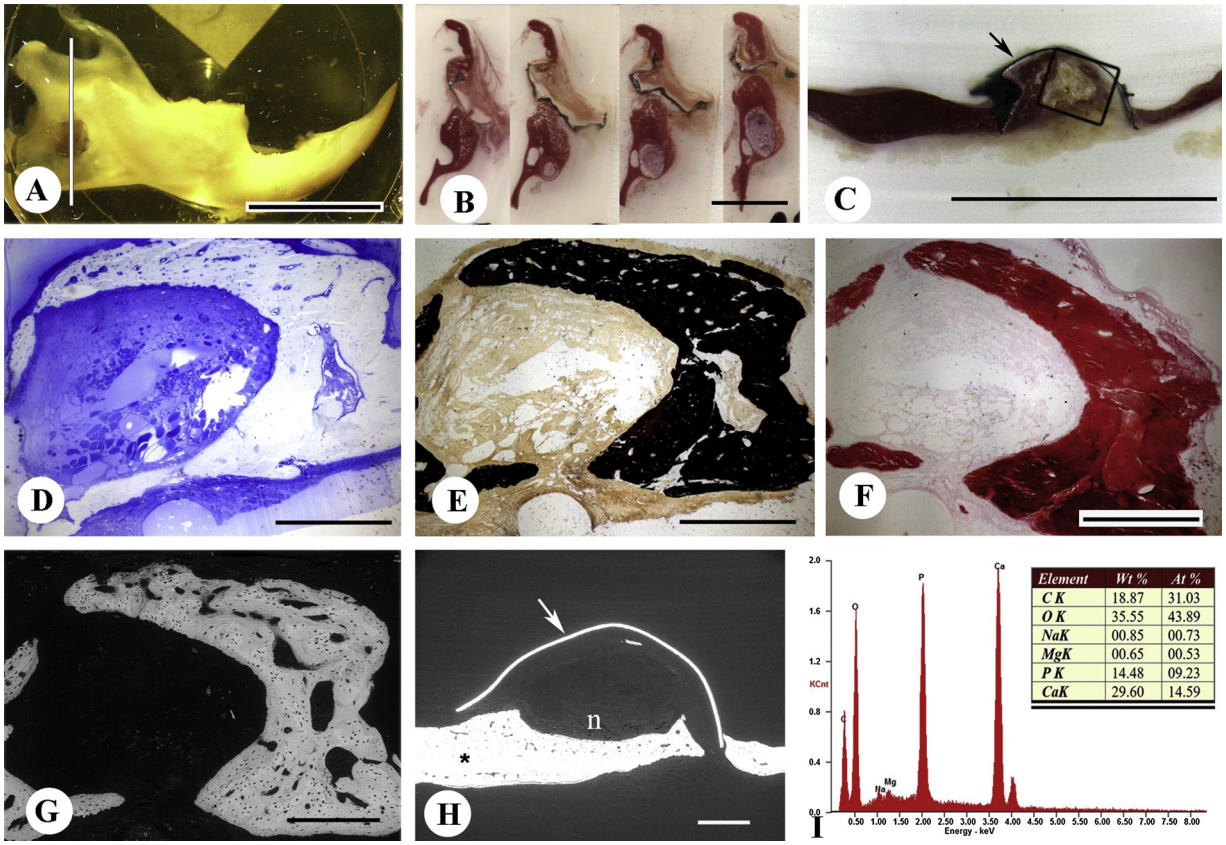

Fig. 2. Different images of a rat mandible processed using the three-sectioning method. **A**: Whole hemimandible embedded in epoxy resin. The vertical white bar indicates the cutting direction. **B**: A short series of thick sections. **C**: In another thick section, an area of interest (black square) was selected to second embedding-sectioning, yielding semithin sections (D-G). Alizarin red. Titanium foil (arrow). Scale bar (A, B and C): 1 cm. **D, E and F**: Different semithin sections under light microscopy stained with toluidine blue (D), von Kossa method (E), and alizarin red (F). **G**: Another semithin section of this series under low pressure SEM. Scale bar (D, E, F and G): 250 mm. **H**: Osteotomy almost closed by titanium foil (white arrow) and bone (n), observed with secondary electron SEM. Scale bar: 500 mm. **I**: Backscattered SEM and EDXS allowed for the elemental analysis of bone from selected area * (H).

samples with a progressively increasing resolution from the naked eye to high power light microscopy and to electron microscopy (Figs. 2 and 3). In the semithin sections, conventional stains are usually not as effective as in the pieces embedded in paraffin, although there are numerous stains useful for sections embedded in epoxy resin. As mentioned earlier, deplasticization can also be performed to improve the penetration of dyes and antibodies. However, we have obtained, without deplasticization, good staining using toluidine blue, alizarin red and von Kossa methods, among others. After deplasticizing with sodium ethoxide, we have also obtained good staining using numerous

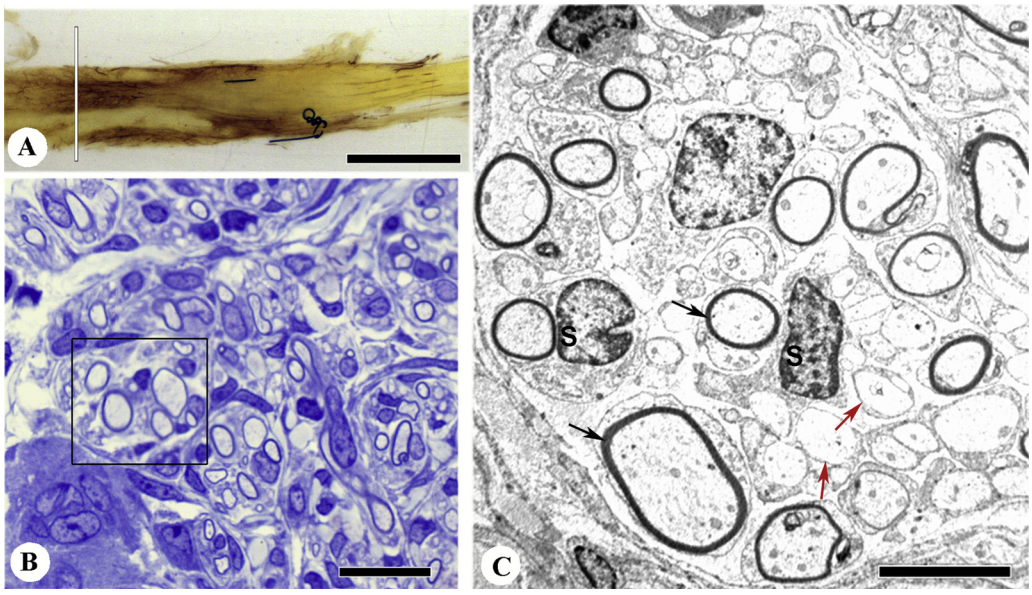

Fig. 3. The three-sectioning method applied to sciatic nerve repair. **A**: Thick section of a decellularized nerve segment. Scale bar: 250 µm. Vertical white bar indicates the cutting direction. **B**: Semithin section of a repairing nerve segment. Scale bar: 20 µm. Toluidine blue. An area of interest (black square) was selected to trim the second embedding block and perform the third-sectioning step, yielding ultrathin sections (C). **C**: Ultrathin section with myelinic (black arrow) and amyelinic (red arrow) nervous fibers supported by Schwann cells (S). Scale bar: 5 µm. Transmission electron microscopy.

techniques, including stains such as haematoxylin, eosin, and safranin.

In addition, we can study these sections with electron microscopy. In thick and semithin sections, it is easy to compare light microscopy results with those obtained from different types of electron microscopy. The secondary electron images are very similar to those obtained from conventional high vacuum SEM and metalized pieces (Fig. 2G and H). Backscattered electron images were found to give a lower contrast compared to the secondary electrons but showed more details of the non-calcified tissues. Since backscattered electrons are sensitive to atomic number, they are also useful for determining phases of different atomic composition and this permits the selection of areas of interest for further analysis using energy dispersive X-ray spectroscopy. For instance, we found the same atomic Ca/P proportion in the neoformed bone as the normal pre-existing bone (Fig. 2I).

When ultrastructural studies are important, it is necessary to perform osmium tetroxide refixation before applying the three-sectioning method. For example, in our study of nerve regeneration (Fig. 3), the whole piece, nerve and graft, was first sectioned to obtain thick sections (Fig. 3A). Areas of interest were selected from this thick section and re-embedded, producing the second embedding blocks. These blocks were cut to obtain semithin sections (Fig. 3B). Target areas identified in the semithin sections (Fig. 3B, black square) allow us to trim the second embedding blocks for cutting them to obtain ultrathin sections. The ultrathin sections were visualized by TEM to assess the progress in neural regeneration (Fig. 3C). If the important details appear only in some semithin sections, these sections can be re-embedded, yielding the third embedding blocks. These third embedding blocks are further cut to obtain the ultrathin sections enclosing these important areas.

As in the case of any other histological method, fixation is one of the determinants for the results obtained and enables the usage of different staining techniques. For example, if the pieces are postfixed with osmium tetroxide, the ultrastructure is better discerned (Fig. 3C), but much immunoreactivity is lost, whereas if we omit the osmium and other heavy metals, the immunoreactivity improves, but the cell membranes are no longer observed.

5. Discussion

We have obtained approximately 20 thick sections (each approximately 300 μm thick) from each centimeter of every piece using this method, and after the second sectioning, several tens of semithin sections (1–3 μm) can be obtained from each thick section. Thus, the entire piece can be explored in the form of thick sections in the search for interesting zones. In these zones, we have the possibility of applying numerous histological techniques on the semithin sections.

The thick sections obtained with our method are easy to handle due to their consistency, hardness, plasticity, and elasticity. As mentioned earlier, this method yields 20 thick sections in the first sectioning from each centimeter of every piece. In comparison, the grinding and polishing method starts by cutting sections whose thickness varies between 100 to 300 μm, the equivalent of our “thick sections”, but one histologic sample of 5 μm in thickness was obtained from each thick section. In contrast, our method can produce, after the second sectioning, several tens of semithin sections (1–3 μm) from each thick section, increasing the possibility of the diversification of histological studies. Additionally, our semithin sections can give better resolution than the thicker ones obtained from the grinding and polishing methods, which cannot manage pieces less than approximately 5 μm in thickness (Malik et al., 1998).

The Spurr´s embedding is easy to prepare, and it is the same technique that is used for transmission electron microscopy. The proportion of the components is measured by weight and clearly established. However, in embedding methods derived from methacrylate, methyl methacrylate, and glycol methacrylate, the proportion of their components is difficult to adjust and usually varies across laboratories. The polymerization reaction of the methacrylate derivatives is exothermic and some of its elements are volatile and potentially dangerous. Recent acrylic embedding methods such as Technovit 9100, ensure that polymerization occurs at low temperatures (-2 to -20 ºC) and they are safer. In contrast, the epoxy resin used in our method is a simple mixture, easy to prepare, that polymerizes in an oven at 60 ºC for 24 h.

It is easier to stain pieces embedded in methacrylate or acrylic plastics than in epoxy resin; however, methodological modifications to stain epoxy resin embedded sections applicable to almost all the common histological methods have been described. Acrylic resins do not form stable bridges, so dyes, chemicals, and antibodies penetrate more easily than in epoxy resins, which form polar bonds. This has been addressed by performing deplasticization with sodium ethoxide (Brorson and Reinholt, 2008) and good results have been obtained in general staining, immunohistochemistry (Tóth and Czoma, 2011), and *in situ* hybridization (Berger et al., 2017; Kitazawa and Kitazawa, 2006; Willbold and Witte, 2010). For example, Tóth and Czoma, 2011 have obtained significant and consistent results using immunohistochemical methods in 936 bone marrow biopsies without decalcification embedded in epoxy resins. In human bone biopsy studies, it has been shown that the non-decalcified bone shows stronger immunoreactivity of characteristic bone markers: osteocalcin, osteonectin, and osteopontin than the decalcified bone, independent of paraffin or resin embedding (Wittenburg et al., 2009).

SEM and elemental analysis can be performed on both the thick and the semithin sections since the region from which the scattered electron X-ray detector carries out its measurements is approximately 400 nm in diameter and depth. Thus, this can be achieved after the first and second sectioning, following our three-sectioning method.

In conclusion, the three-sectioning method is designed to obtain serial sections of hard tissues and large pieces to be studied at different levels of magnification with a wide range of histological techniques. The first embedding-sectioning allows us to study the whole extension of the piece at low magnification and select areas of interest, which are studied using high-resolution light microscopy after the second embedding-sectioning in semithin sections. These are followed by the ultrastructural studies of the interest zone (third embedding-sectioning). The possibility of using several types of electron microscopy significantly facilitates the correlative histology between different histological techniques and enlargements.

Declaration of Competing Interest

Manuel José Gayoso declares that the general fluorescent staining solution used in this study was patented (only for Spain P 200703132) and published by him (Gayoso, 2012).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micron.2020.102841.

References

Bancroft, J.D., Gamble, M., 2008. Theory and Practice of Histological Techniques. Churchill Livingstone.

Berger, C., Helmprobst, F., Chapouton, P., Lillesaar, C., Stigloher, C., 2017. sept8a and sept8b mRNA expression in the developing and adult zebrafish. Gene Expr. Patterns

25–26, 8–21. https://doi.org/10.1016/j.gep.2017.04.002.
Blaauw, E.H., Oosterbaan, J.A., Schakenraad, J.M., 1989. Improved Epon embedding for biomaterials. Biomaterials 10, 356–358. https://doi.org/10.1016/0142-9612(89)90079-3.
Bock, D.D., Lee, W.-C.A., Kerlin, A.M., Andermann, M.L., Hood, G., Wetzel, A.W., Yurgenson, S., Soucy, E.R., Kim, H.S., Reid, R.C., 2011. Network anatomy and in vivo physiology of visual cortical neurons. Nature 471, 177–182. https://doi.org/10.1038/nature09802.
Brorson, S.-H., Reinholt, F.P., 2008. The intensity of immunogold labeling of deplasticized acrylic sections compared to deplasticized epoxy sections-Theoretical deductions and experimental data. Micron 39, 144–150. https://doi.org/10.1016/j.micron.2006.11.006.
Buijs, R., Dogterom, A.A., 1983. An improved method for embedding hard tissue in polymethyl methacrylate. Stain Technol. 58, 135–141.
Caropreso, S., Bondioli, L., Capannolo, D., Cerroni, L., MacChiarelli, R., Condò, S.G., 2000. Thin sections for hard tissue histology: a new procedure. J. Microsc. 199, 244–247. https://doi.org/10.1046/j.1365-2818.2000.00731.x.
Donath, K., Breuner, G., 1982. A method for the study of undecalcified bones and teeth with attached soft tissues. The Säge-Schliff (sawing and grinding) technique. J. Oral Pathol. 11, 318–326. https://doi.org/10.1111/j.1600-0714.1982.tb00172.x.
Gabe, M., 1968. Techniques histologiques. In: Masson Paris 1 (Ed.), Masson et Cie, Paris.
Gayoso, M.J., 2012. MRT letter: a fast and easy method for general fluorescent staining of cultured cells on transparent or opaque supports. Microsc. Res. Tech. 75, 849–851. https://doi.org/10.1002/jemt.22068.
Geith, T., Amarie, S., Milz, S., Bamberg, F., Keilmann, F., 2014. Visualisation of methacrylate-embedded human bone sections by infrared nanoscopy. J. Biophotonics 7, 418–424. https://doi.org/10.1002/jbio.201200172.
Guillaume, O., Geven, M.A., Varjas, V., Varga, P., Gehweiler, D., Stadelmann, V.A., Smidt, T., Zeiter, S., Sprecher, C., Bos, R.R.M., Grijpma, D.W., Alini, M., Yuan, H., Richards, G.R., Tang, T., Qin, L., Yuxiao, L., Jiang, P., Eglin, D., 2020. Orbital floor repair using patient specific osteoinductive implant made by stereolithography. Biomaterials 233. https://doi.org/10.1016/j.biomaterials.2019.119721.
Hudson, T.W., Liu, S.Y., Schmidt, C.E., 2004. Engineering an improved acellular nerve graft via optimized chemical processing. Tissue Eng. 10, 1346–1358. https://doi.org/10.1089/ten.2004.10.1346.
Kammerman, J.R., Prophet, E.B., Barnes, C.F., 1995. Histotecnología ortopédica. In: Prophet, E.B., Mills, B., Arrington, J.B., Sobin, L.H. (Eds.), Métodos Histotecnológicos. American Registry of Pathology, Washington, D. C, pp. 74–75.
Kitazawa, S., Kitazawa, R., 2006. In situ detection of specific gene expression during and immediately after transcription at electron microscopic level. J. Struct. Biol. 153, 64–72. https://doi.org/10.1016/j.jsb.2005.09.010.
Kuhbier, J.W., Weyand, B., Radtke, C., Vogt, P.M., Kasper, C., Reimers, K., 2010. Isolation, characterization, differentiation, and application of adipose-derived stem cells. Bioreactor Systems for Tissue Engineering II. Springer, Berlin Heidelberg, Berlin, Heidelberg, pp. 55–105. https://doi.org/10.1007/10_2009_24.
Lim, J.I., Lee, Y.-K.K., 2009. Oleic acid-added embedding medium for histological analysis of hard tissue. Microsc. Res. Tech. 72, 766–771. https://doi.org/10.1002/jemt.20738.
Majno, G., Joris, I., 2004. Cells, Tissues, and Disease: Principles of General Pathology, 2nd ed. Oxford University Press, New York.
Malik, N., Gunn, J., Holt, C.M., Shepherd, L., Francis, S.E., Newman, C.M., Crossman, D.C., Cumberland, D.C., Newman, M.H., Crossman, D.C., 1998. Intravascular stents: a new technique for tissue processing for histology, immunohistochemistry, and transmission electron microscopy. Heart 80, 509–516. https://doi.org/10.1136/hrt.80.5.509.
Mukhamadiyarov, R.A., Sevostyanova, V.V., Shishkova, D.K., Nokhrin, A.V., Sidorova, O.D., Kutikhin, A.G., 2016. Grinding and polishing instead of sectioning for the tissue samples with a graft: implications for light and electron microscopy. Micron 85, 1–7. https://doi.org/10.1016/j.micron.2016.03.005.
Palay, S.L., Chan-Palay, V., 1974. Cerebellar Cortex: Cytology and Organization. Springer, Berlin. https://doi.org/10.1007/978-3-642-65581-4.
Rippstein, P., Black, M.K., Boivin, M., Veinot, J.P., Ma, X., Chen, Y.-X., Human, P., Zilla, P., O'Brien, E.R., 2006. Comparison of processing and sectioning methodologies for arteries containing metallic stents. J. Histochem. Cytochem. 54, 673–681. https://doi.org/10.1369/jhc.5A6824.2006.
Rousselle, S.D., Wicks, J.R., Tabb, B.C., Tellez, A., O'Brien, M., 2019. Histology strategies for medical implants and interventional device studies. Toxicol. Pathol. 47, 235–249. https://doi.org/10.1177/0192623319827288.
Savi, F.M., Brierly, G.I., Baldwin, J., Theodoropoulos, C., Woodruff, M.A., 2017. Comparison of different decalcification methods using rat mandibles as a model. J. Histochem. Cytochem. 65, 705–722. https://doi.org/10.1369/0022155417733708.
Sennerby, L., Thomsen, P., Ericson, L.E., 1993. Early tissue response to titanium implants inserted in rabbit cortical bone - part I ligth microscopic observations. J. Mater. Sci. Mater. Med. 4, 240–250. https://doi.org/10.1007/BF00122275.
Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31–43. https://doi.org/10.1016/S0022-5320(69)90033-1.
Tóth, Z., Czoma, V., 2011. Immunohistochemistry on epoxy resin-embedded bone marrow biopsy experience with 936 cases. Appl. Immunohistochem. Mol. Morphol. 19, 15–20. https://doi.org/10.1097/PAI.0b013e3181ec005e.
Velde, J., Burkhardt, R., Kleiverda, K., Leenheers-Binnendijk, L., Sommerfeld, W., 1977. Methyl-methacrylate as an embedding medium in histopathology. Histopathology 1, 319–330. https://doi.org/10.1111/j.1365-2559.1977.tb01671.x.
Willbold, E., Witte, F., 2010. Histology and research at the hard tissue-implant interface using Technovit 9100 New embedding technique. Acta Biomater. 6, 4447–4455. https://doi.org/10.1016/j.actbio.2010.06.022.
Willbold, E., Reebmann, M., Jeffries, R., Witte, F., 2013. Electrochemical removal of metallic implants from Technovit 9100 New embedded hard and soft tissues prior to histological sectioning. Histochem. Cell Biol. 140, 585–593. https://doi.org/10.1007/s00418-013-1089-6.
Wittenburg, G., Volkel, C., Mai, R., Lauer, G., 2009. Immunohistochemical comparison of differentiation markers on paraffin and plastic embedded human bone samples. J. Physiol. Pharmacol. 60 (Suppl 8), 43–49.