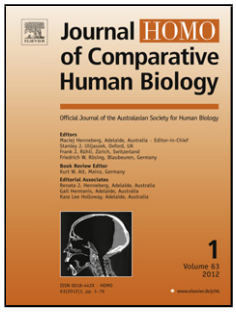
A revised method for the preparation of dry bone samples used in histological examination

Five simple steps



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PII: S0018-442X(17)30040-9
DOI: http://dx.doi.org/doi:10.1016/j.jchb.2017.07.001
Reference: JCHB 25480

To appear in:

Received date: 3-11-2016
Accepted date: 10-7-2017

Please cite this article as: García-Donas, J.G., Dalton, A., Chaplin, I., Kranioti, E.F.,A revised method for the preparation of dry bone samples used in histological examination: five simple steps, *Journal of Comparative Human Biology* (2017), http://dx.doi.org/10.1016/j.jchb.2017.07.001

A revised method for the preparation of dry bone samples used in histological examination: five simple steps

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Received 3 November 2016, accepted 10 July 2017

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Abstract

Histology of dry bone tissue has many scientific applications. The histological analysis of bone requires the production of good quality thin sections. Many researchers have developed new histological techniques and/or they have refined existing ones. In this paper, we describe a revision of histological techniques for obtaining thin sections from modern dry bone. The method is easy to apply and the equipment required is commonly found in a histology laboratory. In comparison to other techniques presented in the literature, this adapted method reduces the number of consumables and steps, thereby improving the efficiency and cost-effectiveness of the procedure.

Introduction

Histological methods are a potential means for the examination of human skeletal remains. The incorporation of histomorphometry in the analytical routine provides a new insight that cannot be obtained through the macroscopic observation of bone structures (Ortner, 1975). Due to this, bone thin sections are used extensively in both animal and human research for a variety of purposes (Chinsamy and Raath, 1992; Hillier and Bell, 2007). Bone microscopy assessment is done in a wide range of research, such as ontogeny (Chinsamy, 1995; Goldman et al., 2009), comparative anatomy (Cvetkovic et al., 2013; Singh et al., 1974), human osteology (Cuijpers, 2006), biological and forensic anthropology (Kim et al., 2007; Pfeiffer et al., 2006) and diagenesis studies (Bell, 2011; Kontopoulos et al., 2016), to name just a few. Thus, the production of readable thin sections for these diverse research purposes is an essential requirement.

Methodological aspects for the elaboration of bone thin sections have been covered and revised by many studies (e.g. Iwaniec et al., 2008; Simmons, 1985). Early techniques, such as that proposed by Frost in the 1950s for the preparation of thin sections of undecalcified fresh bone tissue comprised the use of basic equipment and an effective process based on a manual procedure (Frost, 1958). In recent years, researchers have modified the original method by incorporating new products or improving the specimens by staining the samples (de Boer et al., 2013; Maat et al., 2001). Regarding methods that use more specialised equipment and a greater variety of consumables, specific training may be required in order to follow the procedural steps (Caropreso et al., 2000).

The goal of this paper is to present an improved technique to produce histological thin sections of undecalcified dry bone. The method proposed – developed in collaboration with an expert in the preparation of rock thin sections – is primarily based upon a technique used in ceramic petrography. Although there exists no single standard technique for preparing petrographic thin sections, a situation that is a legacy of the interdisciplinary nature of its development, the existing one adapted for the present study is in common use (Quinn, 2013).

Materials and methods

The skeletal series used for this study consisted of ten bones (normal ribs and metatarsals) obtained from the Cretan Collection, a modern osteological collection from Crete (Greece) (Kranioti et al., 2008; Kranioti and Michalodimitrakis, 2009). The age of the specimens ranged from 6 to 90 years in order to test whether the thickness of the cortical bone affects the performance of the method. Unless stated otherwise, Buehler equipment and consumables are used throughout (Buehler, Esslingen am Neckar, Germany). Due to the nature of the sample, dry bone was used. No additional steps to dry the specimens before histological preparation were required.

Cutting a segment

If the rib is complete, a small piece of approximately 20-40 mm in length – depending on the number of thin sections that need to be extracted from the selected specimen– has to be cut from the rib. In the present study, a Dremel 3000 variable speed multi-tool fitted with a diamond-cutting wheel is used.

Embedding

The samples are encapsulated in epoxy resin (EpoThin 2® epoxy system is used) in order to provide support and maintain their integrity during the thin sectioning process. The bone samples are arranged in moulds of appropriate size, placed into a mounting/embedding system (a Cast N' Vac 1000 vacuum impregnation system) and the resin mixture poured into the moulds. The resin is impregnated into the bone sample under vacuum; to ensure that the resin penetrates into any voids existing within the sample without the formation of bubbles. It is recommended to repeat the cycle of evacuation several times to ensure that any pockets of air have been removed and the

bone is completely impregnated by the resin. After this process, the samples need at least 24 hours to cure.

Once the resin is cured, the sample can be removed from the mould and prepared for mounting onto a glass slide. If the bone sample is longer than 10 mm then it can be reduced to a more manageable size. This is done in the present study by mounting the sample on a single saddle chuck attached to an IsoMet 1000 precision saw and cut using a 15LC diamond wafer blade (blade thickness 0.5 mm). In order to remove any sharp edges formed by the encapsulation process or burrs to the resin caused by the cutting blade, the embedded sample needs to be ground on a grinder-polisher. An UltraPrep 20 μm diamond abrasive disc fitted to a MetaServ 250 equipped with a Vector 250 power head is used for this purpose in the present study. This process takes only a few seconds. (NB: if there are still voids within the trabeculae on the exposed surface of the encapsulated sample, it is advisable to introduce a small quantity of prepared resin using a syringe and leaving to cure once again. These voids can cause breaks on the surface while grinding the sample (see below) with the risk of losing some of the bone surface.) The surface of the sample that is going to be bonded to the glass slide must be optically flat and devoid of scratches; as this is the surface that will be in direct contact with the slide. Any remaining imperfections cannot be eradicated once mounted. Removal of imperfections is done by grinding and polishing the surface to be bonded using silicon carbide abrasive discs fitted to the grinder-polisher. A fairly aggressive grit size is used initially (CarbiMet P1200 [FEPA]) to reduce the more pronounced imperfections, before moving onto a finer grade (MicroCut P2500 [FEPA]). The final polishing stage is performed using a MicroCut P4000 disc.

Mounting

The sample is now ready to be mounted to the glass grinding slide, but before this can be done, the surface of the slide needs to be frosted, or lapped, to create a better surface for bonding. Ready-made frosted glass grinding slides can be purchased or, as in this paper, plain grinding slides can be frosted by hand. This is a quick and simple process whereby a paste of abrasive powder (in this instance, Logitech 15 μm calcined aluminium oxide powder) and water is prepared on a glass plate. The surface of the glass slide is then ground for approximately 1 minute on the glass plate, with the paste forming the abrasive that creates the frosted effect. The slide should then be cleaned by soaking in either acetone or soapy water to remove any residue and left to dry.

The encapsulated sample is bonded to the frosted surface of the glass slide by spreading a small quantity of resin over the prepared surface of the sample and applying even pressure to ensure a firm bond. If possible, a weight, ideally by means of a bonding jig, is placed on the sample to ensure that it remains in contact with the surface of the slide rather than floating on the freshly applied resin.

Sectioning

The slide is secured in a suitable glass slide chuck (for the present study, a chuck for holding 27 × 46 mm slides is used) which is mounted on the precision cutting saw. The chuck is then positioned so that all but 1 mm of the encapsulated sample is cut away from the glass slide, thus leaving a 1 mm thin section of bone bonded to the slide. This was done with the cutting blade rotating at a speed of 225 rpm, taking approximately 2-3 minutes, depending on the size of mould used for encapsulating the sample.

Final grinding and polishing

The next step consists of grinding the thin section to remove scratches caused by the cutting blade using the same process and grades of silicon carbide abrasive discs mentioned above in step 2. This is done by holding the slide in a petrographic glass slide holder. The thin section should be ground down to a thickness of approximately 70-50 μm, an amount that will allow the observer to see the microstructures through a transmitted or polarizing light microscope.

Finally, a cover slip can be applied to the prepared sample by using a small quantity of epoxy resin as a bonding agent, which is allowed to set. A sketch of the five steps, necessary equipment and consumables used is shown in Figure 1**.**

INSERT Fig. 1 ABOUT HERE

Results

The application of the revised histological method on the ten bones resulted in the production of high-quality thin sections which are comparable to the results produced by other studies in the literature (Beauchesne and Saunders, 2006; Paine, 2007). The histological features were intact and they could be observed clearly under 4×, 10× and

40× magnifications with excellent clarity (both transmitted and polarised light microscope) (Fig.2). The integrity of the bone – independently of the thickness of cortical area – was in all cases preserved without losing part of the sample through the grinding process. For one of the slides, the bone appeared to be blurred when observed under the microscope due to the resin bond between the bone and the frosted glass slide lifting. This minor technical setback was remedied by adding slightly more resin when mounting the resin block onto the frosted slide (see step 3 of the Materials and methods section). No other technical issue was observed following this adjustment.

INSERT Fig. 2 ABOUT HERE

Discussion

Bone histology has been used for many research purposes, from the study of bone development to the assessment of taphonomic processes (Jasinoski and Chinsamy, 2012; Turner-Walker and Jans, 2008). As the purpose of this paper is not to review all research goals achieved by bone histology but to propose a technical approach to the preparation of the samples, the reader is referred to Hillier and Bell (2007) for further research applications. The preparation of histological thin sections is a non-standardised procedure that requires the use of specific and expensive equipment and consumables; some techniques which use less products and/or equipment have been proposed elsewhere (e.g. Maat et al., 2001). With the current adapted technique, we present an alternative process that reduces the variety of consumables and the number of steps (Fig. 1). Therefore, the method presented here is a combination of shared steps followed by different published research on histological preparation of bone samples (Caropreso et al., 2000; Paine, 2007; Schultz, 2003; Tiesler et al., 2006). The method most similar to ours (Chinsamy and Raath, 1992) was published a quarter of a century ago. The innovation of better laboratory consumables and equipment has taken place during the intervening years.

Compared to similar methods found in the literature, the steps and consumables proposed in this paper are equivalent but more time efficient while producing thin bone sections of equal quality and reducing the number of consumables required (Maat et al., 2001; Paine, 2007; Stout and Paine, 1992). For example, the same epoxy resin is used

for encapsulating the sample and as a bonding agent in the thin section preparation process. Thus, the stage of bonding the wafer onto the glass slide with mounting media such as e.g., Permount® is avoided, as is the time required for it to dry out (Crowder et al., 2012; Paine, 2007). Maat and his colleagues' (2001) technique suggested that a disadvantage in using resin was the length of time taken for it to cure. The time required for the preparation of the sections will vary depending on the type or brand of resin used during the embedding process. Biodur® resin is used in a number of published methods (Schultz, 2003; Schultz, 2012; Turner-Walker and Mays, 2007; Pavón et al., 2010) due to the suitability of its characteristics for producing stable, good quality thin sections. This particular resin, however, generally takes at least three weeks to cure, and although Haas and Storå (2015) devised a method to reduce this time to a length similar to that prescribed for the resin used in the present study, they did so by means of a more laborious process. In the present study, the use of Buehler EpoThin® resin demonstrated, not only a reduction in time, but also, and most importantly, thin sections of equal quality. A major advantage in the use of Biodur® is the fact that it penetrates the bone sample effectively, thus offering a good sample stability and minimising any potential incidences of the thin section from "lifting" the bone from the microscope slide (Schultz, 2003; Schultz, 2012). This paper did not compare the two types of resin. Nonetheless, it would be a further step in the refinement of this revised method to test whether the lifting issue experienced with some of the thin sections could be overcome by using another type of resin. So far, there are two stages in our method that seem crucial for avoiding this matter: firstly, full penetration of the trabecular area with resin (see embedding section for details) before cutting the thin section in order to avoid air or water penetrating into the sample; and secondly, placing a sufficient amount of resin during the mounting process to ensure that the space between the grinding slide and the sample is filled completely by the agent. The other advantage of resin being used as a mounting agent is that the preservation of the sample appeared to increase. In contrast, when using the glue for mounting the thin section onto the glass slide, lifting and disintegration of the thin section occurred. This has been noted elsewhere (Beauchesne and Saunders, 2006; Haas and Storå, 2015), pointing out that cyanoacrylate glue used in some methods (de Boer et al., 2013; Maat et al., 2001) is not suitable for fragile specimens. The use of resin instead of Permount® or any other adhesive as a surrogate mounting agent prevents the bone from falling apart even for thin cortical areas (as often happens in old specimens). Moreover, we verify that there is no need to use

another type of resin for mounting the samples on the glass slides as it was done by other authors (Beauchesne and Saunders, 2006; Chinsamy and Raath, 1992). This fact was corroborated by Haas and Storå (2015), who used the same resin for embedding and mounting. Their explanation focused on exothermic processes being the cause of tension created between the sample and the slide resulting in shrinkage and lifting of the thin section. Moreover, other methods, such as Tiesler et al. (2006), used the same resin for embedding and mounting, although their technical approach is more time consuming due to the time required for the resin to cure and the application of several layers of resin until the surface is fully prepared. In the proposed method, no heating is necessary for speeding up the curing process and considering that the length of time proposed by other methods (Haas and Storå, 2015) is equivalent to the one spent here, possible drawbacks of increase tension are avoided. As the EpoThin® resin used achieves a peak exotherm of 40ºC during the curing process, these issues were not evident for the present revised method.

As demonstrated by other techniques (Beauchesne and Saunders, 2006; Chinsamy and Raath, 1992), the option of mounting the resin block on the grinding glass prior to extracting the thin section provides a more stable base for the thin section as it is always firmly supported. This is in contrast to other methods that first extract the thin section followed by mounting on a glass slide (Tiesler et al., 2006), which could result in shrinkage or lifting, as it was experienced previously by one of the authors.

As the authors produced two sets of sections by applying Paine's (2007) method and the revised version, it seems reasonable to highlight the differences between the two techniques. Regarding the frosting of the slide, using aluminium oxide abrasive powder is less labour intensive (also applied by for example, Tiesler et al., 2006), and it allows an even, frosted surface (Chinsamy and Raath, 1992). The reader is reminded that slides can be obtained, pre-frosted, from a number of manufacturers. As can be seen in Figure 2, the quality of the images does not differ from the Paine (2007) technique to the one proposed in this paper.

Our method can be applied to bones other than ribs or metatarsals by adjusting the size of the microscope slide and the slide chuck to the bone to be sectioned. Nonetheless, there is a need of further research on these larger bones for which it would be worth considering the use of other type of resin for encapsulation, e.g., Biodur® - due to its greater penetration of porous materials - or for mounting to the glass slide. That being said, in a separate, preliminary investigation using nineteenth-century

archaeological femora from Blackburn (Lancashire, UK) undertaken by two of the authors, the resin used in the proposed revised method did occasionally pose problems regarding lifting of the thin section. However, the simple expedient of substituting glass slides with acrylic ones rectified this, as recommended by Timothy Bromage (personal communication). The slightly greater flexibility offered by acrylic slides compared to glass ones could reduce the potential for thin sections to lift as a result of the tension caused by exothermic processes during the polymerization of the resin when used as a mounting agent.

Despite the fact that the proposed revised method was not tested on teeth, other authors used similar methods on dental hard tissue with satisfactory results (Caporeso et al., 2000). This suggests that our method may be suitable for dental material as well. In addition, the technique is applicable to both archaeological and modern material. In the latter case, maceration may be needed (Cho, 2012).

In conclusion, this paper presents a modification of pre-existing methods for the preparation of thin sections of bone as developed by our research group. We found this technique easier and the results more consistent in terms of quality of the thin sections compared to previous methods we tried (e.g. Paine 2007). A simplification of steps and the limitation of consumables are achieved making the process more practical and less time-consuming. It is understandable that every laboratory has its own protocols that are developed according to the available equipment, budget restrictions and personnel's background and training. Thus we are not aiming to propose this method as the most suitable but simply to offer an alternative way of preparing thin sections. Last, it must be stressed that despite the fact that the method is explained in detail and is relatively easy to apply, special training and specific equipment are required for following the steps described in this paper.

Acknowledgments

Alan Dalton and Elena Kranioti were funded by the SHCA of the University of Edinburgh for the specialised training in histological techniques that took place at the University of Durham. Special thanks to Effrosyni Michopoulou for the thin section of the metatarsal bones and to Dr. Robert R. Paine for the histological training on his methodology. Many thanks to Prof. Timothy Bromage for the correspondence and advice on processing thin section with larger bones.

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Legends for figures

Fig. 1. Summary of the procedure, equipment and consumables used in the present technique.

Fig. 2 (colour reproduction). Examples of eight photomicrographs taken using a research microscope (Leica DM750P equipped with a Leica MC170 HD camera); *(A)* and *(B)* rib section processed using Paine (2007) method (40× and 100× respectively); Revised method was used for; Ribs (*C)*, (*D)* and (*E)* (40×, 100× and 500× respectively); *(F)* pathological rib specimen (periostitis; 100×); *(G)* and *(H)* metatarsal (cortical area and 40×).

Fig. 1.

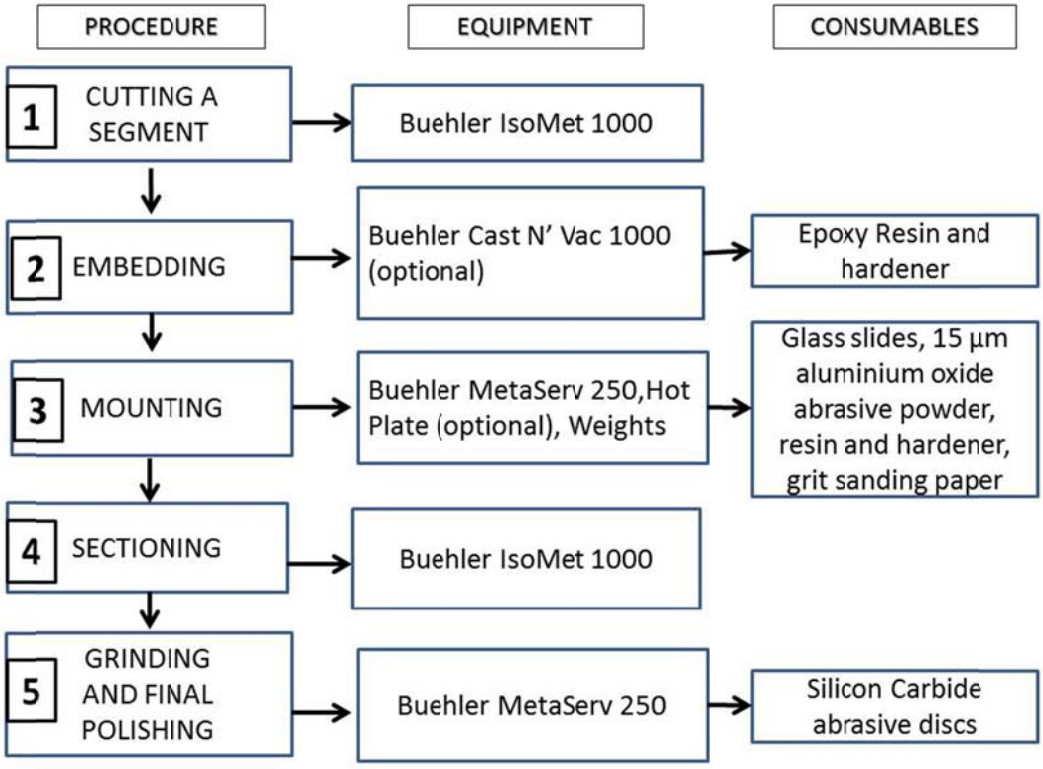

Fig. 2.