REVISED METHOD FOR THE PREPARATION OF DRY BONE SAMPLES USED IN HISTOLOGICAL EXAMINATION: five simple steps.

García-Donas, J.G.[a*]; Dalton, A.[a]; Chaplin, I.[b]; Kranioti, E.F.[a]

[a] Edinburgh Unit for Forensic Anthropology, School of History, Classics and Archaeology, Archaeology Department, University of Edinburgh, Edinburgh, Scotland, UK.

[a] Department of Earth Sciences, University of Durham, Durham, England, UK.

*** Corresponding author: Julieta G. García-Donas, tel: 07858051060, email: julietagomezgd@gmail.com**

Abstract

Histology of dry bone tissue has many scientific applications. The histological analysis of bone requires the production of 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 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, 1970). Due to this, bone thin sections are used extensively in both animal and human research for a variety of purposes (e.g. Chinsamy & Raath, 1992; Hillier & Bell, 2007). Bone microscopy assessment is done in wide range of research, such as ontogeny (Chimsamy, 1995; Goldman et al., 2009), comparative anatomy (Hill, 2006; Cvetkovic et al., 2013), human-osteology (Cuijpers, 2006), forensic anthropology (Pfeiffer et al., 2006; Kim et al., 2007) and diagenesis studies –(Bell, 2008; 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 in order to facilitate these fields of research.

Methodological aspects for the elaboration of bone thin sections have been covered and revised by many studies (e.g. Simmons, 1985; Iwaniec, Wronski & Turner,

2008). 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 elucidating the specimens by staining the samples (Maat, Van Den Boss & Aarents, 2001; De Boer, Aarents & Maat, 2013). Regarding methods that utilise more specialised equipment and a greater variety of consumables, specific training may be required in order to follow the procedural steps (e.g. 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 utilized 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: 9, 23-29).

MATERIALS AND METHODS

The sample used for this study consisted of ten bones (standard ribs and metatarsals) obtained from the Cretan Collection, a modern osteological collection from Crete (Greece) (more information can be found in Kranioti, Iscan & Michalodimitrakis, 2008; Kranioti & Michalodimitrakis, 2009). The age of the specimens ranged from 6 to 90 years of age in order to test whether the thickness of the cortical area 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) no previous steps to dry the specimens was required.

1. Cutting a segment.

If the rib is complete, a small piece of approximately 2-4 cm in length – depending on the number of thin sections that need to be extracted from the selected specimen– has to be cut from the remaining length of rib (in the present study a Dremel 3000 variable speed multi-tool fitted with a diamond cutting wheel is used).

2. 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 can be left overnight 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 1 cm 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

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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. This 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.

3. Mounting.

The sample is now ready to be mounted to the glass 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 slides can be purchased or, as in this paper, plain 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 detergent in 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. Additionally, the slide can be heated –just leaving the frosted slide on a hot plate to approximately 60°C- prior to bonding to further reduce any possibility of floating. The heating also reduces the length of time required for the resin to cure. Once the resin used to bond the sample to the glass slide is cured (preferably leaving overnight), the sample is ready to be thin sectioned.

4. Sectioning.

The slide is secured in a suitable glass slide chuck (for the present study, a chuck for holding 27 × 46 mm slides) 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.

5. 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 are shown in **Figure 1.**

Figure 1 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 (see, for example, Beauchesne & Saunders, 2006; Paine, 2007). The histological features were intact and they could be observed clearly under 4×, 10× and 40× magnification with excellent clarity (both transmitted and polarised light microscope) (***Figure 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 methodological adjustment.

Figure 2 here.

DISCUSSION

Bone histology has been used for many research purposes, from the study of bone development to the assessment of taphonomic processes (Jasinoski & Chinsamy, 2012; Turner-Walker and Jans, 2008). 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 utilise less products and/or equipment have been proposed elsewhere (Maat, Van Den Boss & Aarents, 2001). With this adapted technique, the authors present an alternative process that reduces the variety of consumables and the number of steps (see **Figure 1**).

Compared to similar methodologies found in the literature, the one proposed in this paper is more time efficient, with equal quality in the production of bone thin sections and a reduction of the number of consumables required (Stout & Paine, 1992; Paine, 2007; Chinsamy & Raath, 1992). For example, epoxy resin is used for encapsulating the sample and as a bonding agent in every stage of the thin section preparation process. Thus stage of bonding the wafer onto the glass slide with Permount is avoid. In so doing, the need to use this agent is obviated and the time required for it to dry out (Paine, 2007; Crowder, Heinrich & Stout, 2012). In comparison with Paine (2007), the frosting technique is less labour intensive as the frosting of the slide is carried out by using aluminium oxide abrasive powder which allows an even frosted surface. The glass slide surface has a smooth and clean appearance which removes the step of mounting the thin section onto the reading microscope slide. Therefore, it is a more efficient process. As can be seen in ***Figure 2***, the quality of the images does not vary from the mentioned technique to the one proposed in this paper. Moreover, while using the methodology suggested by Paine (2007), one of the authors experienced problems as parts of the thin section of bone disintegrated during the final grinding process; this problem has been reported on by other researchers as well (Beauchesne & Saunders, 2006). With our adapted technique, the use of resin instead of Permount or any other adhesive as a mounting agent prevents the bone from falling apart even for thin cortical areas (as often happens in old specimens). This is probably due to the fact that resin becomes a hard and

fairly inflexible substance when cured and therefore provides a more robust support for the thin sections of bone.

Another advantage of our method is that after the preparation of the first thin section, the bone sample embedded in its resin block is ready to be used for creating additional slides by repeating the grinding, bonding and sectioning steps in less time than other proposed techniques (e.g. Paine, 2007; Crowder, Heinrich & Stout, 2012).

Our method can be applied to other bones apart from ribs or metatarsals by adjusting the size of the glass slides and the glass slide chucks to the bone to be sectioned (e.g. femur). Although it was not tested on teeth, other authors used similar methodologies on dental hard tissue with satisfactory results (Caporeso et al., 2000). There is also the possibility of using fresh bone or archaeological specimens following exactly the same steps described here (for maceration of bone see Cho, 2012).

Although the technique is explained in detail and easy to apply, special training and specific equipment are required for following the steps described in this paper. Nonetheless, a simplification of both steps and consumables is achieved.

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FIGURES:

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

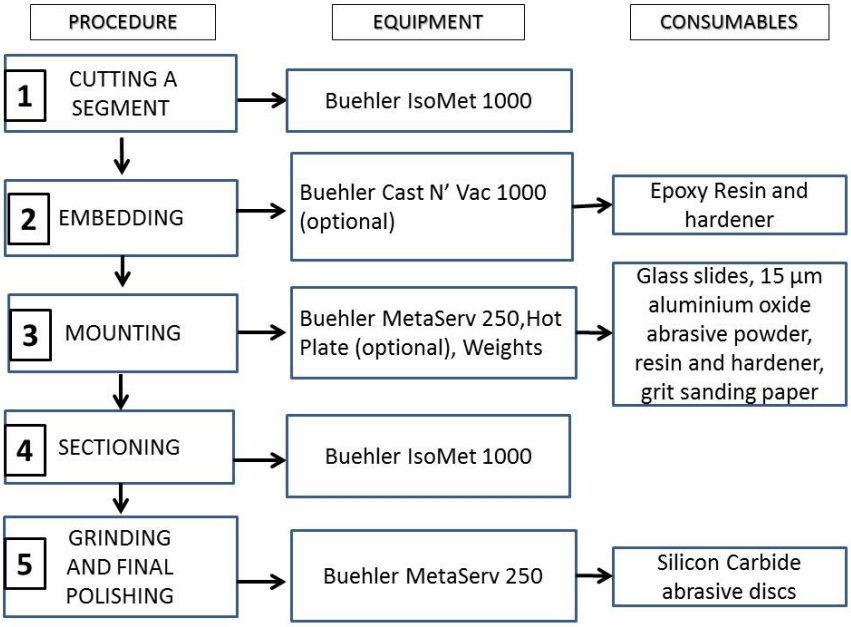
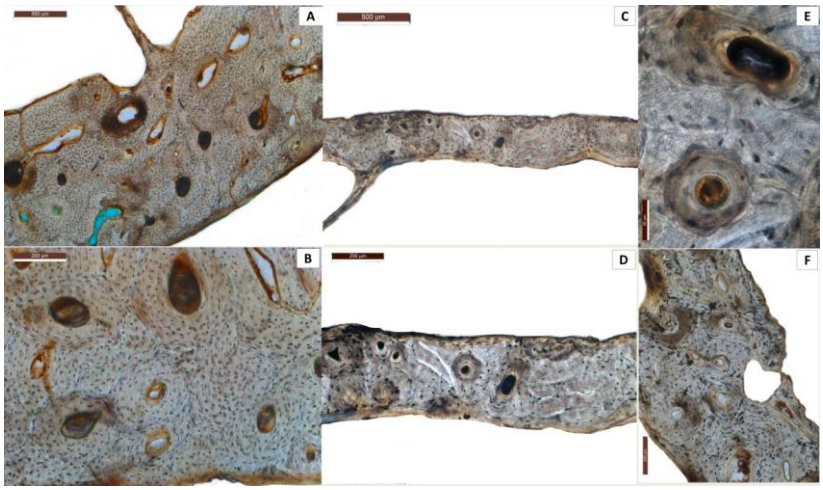
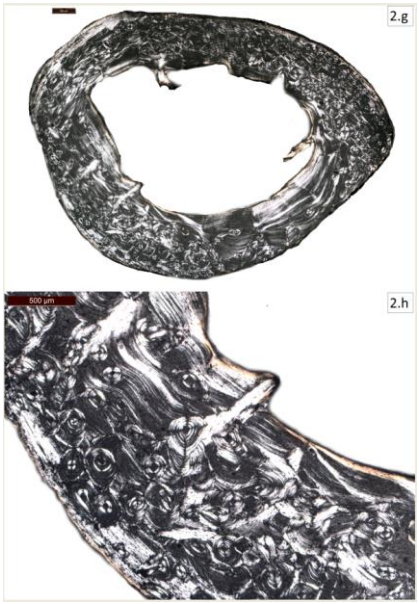


Figure 2 (color reproduction).

Examples of eight photomicrographs taken with a research microscope (Leica DM750P equipped with a Leica MC170 HD camera). *(A)* and *(B)* rib sections are processed using the method offered by Paine (2007) (40× and 100× respectively). Our method is used to produce ribs (*C)*, (*D)* and (*E)* (40×, 100× and 500× respectively); *(F)* pathological rib specimen (periostitis; 100×); *(G)* and *(H)* metatarsal (cortical area at 40×).

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