Annals of Anatomy 222 (2019) 153–157

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.com/locate/aanat

Histological comparison between laser microtome sections and ground specimens of implant-containing tissues

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ARTICLE INFO

Article history:
Received 20 June 2018
Received in revised form
13 November 2018
Accepted 3 December 2018

Keywords:
Cutting-grinding technique
Laser microtomy
Bone histology
Zirconia implants
PHB implants

ABSTRACT

Evaluation of bone regeneration and peri-implant bone apposition can only be accomplished using laboratory techniques that allow assessment of decalcified hard tissue. It is known that 5–15 μm thick sections can be prepared with the cutting-grinding technique, but their production causes a high material loss (≥0.5 mm) between two sections and requires years of training and experience. With the development of the laser microtome it has become possible to cut decalcified bone without high sample material loss. Many scientific publications deal with the application possibilities of the individual methods So far, there is no comparison work between the cutting-grinding technique and laser microtomy. For this reason, new tissue sections were prepared by laser microtome and analyzed histologically from samples that had been previously been prepared by the cutting-grinding technique. Using both methods, it could be demonstrated that the different implants were completely surrounded by a connective tissue layer. In sections (50–100 μm) produced by the routine cutting-grinding technique, magnifications up to 20× revealed no detailed histological information because cell structures could not be clearly identified. By contrast, laser microtome sections (10 μm) revealed these information as e.g. osteocytes are already clearly visible at 10× magnification. Furthermore, the interface between implant and the surrounding bone could be clearly demonstrated due to visible demarcation between a capsule and connective tissue. At the histological level, laser microtome sections were clearly superior at thicknesses ≥30 μm compared to sections produced by the cutting-grinding technique. In addition, laser microtomy has the advantages of time saving and markedly reduced sample loss, especially in cases of the production of serial sections.

1. Introduction

For the study of implants and their impact on the surrounding bone tissue, it is very important to preserve both the inorganic and the organic structures (implant-tissue interface). For this purpose, thin tissue sections must be prepared, stained and analyzed under a light or fluorescence microscope. In addition, thin sections are needed to identify occurring inflammatory reactions such as giant cells or encapsulations of the implants. Usually, such sections are produced by means of a microtome, which achieves layer thicknesses of 3–5 μm. For bone or teeth, the tissue has to be decalcified otherwise it cannot be cut by a conventional microtome using normal paraffin histology. Decalcified sections in the preparation are known to have the disadvantages of curling, wrinkling, slice thickness variations (Cano-Sanchez et al., 2005) and loss of tissue information (e.g. differentiation between mineralized and non-mineralized bone tissue). In addition, decalcification, depending on the material, is very time consuming and can take up to 4 weeks (for example in teeth).

The cutting-grinding technique offers a good option, as the implant-containing bone samples do not have to be decalcified and the relevant areas can be structurally preserved (Donath and Breuner, 1982). First, the samples are embedded in plastic instead of paraffin (e.g. methylmethacrylate). The resulting plastic block is glued by means of a vacuum press on both sides, each with a plexiglas slide, clamped in the cutting-off system and a 100–200 μm thick section is separated by the use of a high precision diamond saw. This thick section is partially ground, honed and polished to a thickness of about 50 μm with a high precision grinding machine (Donath and Breuner, 1982; Rohrer and Schubert,

☆This paper belongs to the special issue Dentistry 2019.
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https://doi.org/10.1016/j.aanat.2018.12.001
0940-9602/

1992; Lang, 2006). It is well known that he preparation of sections of about 5–15 μm of thickness can be prepared by using this cutting-grinding technique (Donath and Breuner, 1982; Rohrer and Schubert, 1992), but this is very difficult and requires years of training and experience. A disadvantage of this cutting technique is the high loss of material of the sample of more than 0.5 mm between two sections (Will and Richter, 2015).

With the development of a new sectioning device, the laser microtome, it has become possible to cut non-decalcified bone without too much material and to prepare serial sections of bone-implant-samples with a thickness between 10–100 μm (Lubatschowski, 2007). Laser microtomy is a procedure for non-contact cutting of samples. In contrast to mechanically working microtomes, an ultrashort pulsed laser (femtosecond laser) serves as a cutting tool. The laser emits radiation in the near infrared range. In this wavelength range, the laser can penetrate biological tissues, but also other materials, to a certain depth without visible damage (Lubatschowski, 2007; Lubatschowski et al., 2012; Will and Richter, 2015).

Many scientific publications deal with this preparation method and its applications (Rohrer and Schubert, 1992; Cano-Sanchez et al., 2005; Lubatschowski, 2007; Lubatschowski et al., 2012; Mai et al., 2012; Gredes et al., 2015; Will and Richter, 2015; Boyde, 2018). So far, however, there is no comparative work on the histological structures visible in specimens prepared by either the cutting-grinding technique or the laser microtome. For this reason, we compared results of histological analyses of tissue sections that were prepared by means of laser microtomes from samples that had already been examined by the cutting-grinding technique (Mai et al. 2012; Gredes et al. 2015).

2. Materials and methods

2.1. Cutting-grinding technology

All used samples with zirconia or PHB implants were archival and had been used in previous studies (Mai et al., 2012; Gredes et al., 2015). For our investigations they had been fixed in 4% PBS-buffered formalin, dehydrated in a graded series of alcohol, and embedded in methyl-methacrylate (Technovit 9100 neu, Kulzer, Germany). Sections of the Technovit-embedded specimens were prepared using a diamond saw and thick sections were ground down to a thickness of 50 μm (for PHB) or 100 μm (for zirconia implants) with a high precision grinding machine. Before staining with hematoxylin/eosin (HE) or Masson-Goldner trichrome, the sections were etched with acetone/96% ethanol (1:1) for 30 s and then watered.

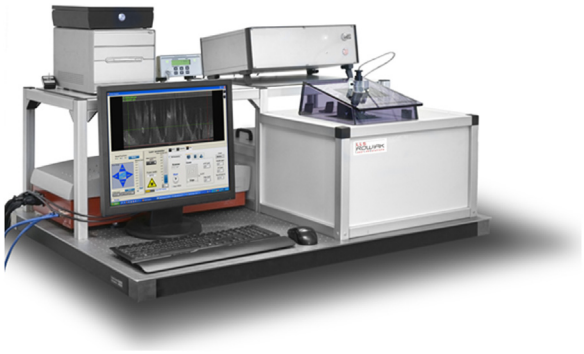

Fig. 1. Lasermicrotome TissueSurgeon.

2.2. Laser microtomy

Prior to staining, serial longitudinal sections of non-decalcified specimens were prepared using a laser microtome (Fig. 1) to a thickness of approximately 10 μm (TissueSurgeon, LLS ROWIAK LaserLabSolutions, Hannover, Germany) as described by Lubatschowski (2007) as well as by Will and Richter (2015). The laser microtome is an inverted optical system, using a near infrared femtosecond laser for optical cutting of plastic embedded specimen for histology (Fig. 2). The sections were deplastified and rehydrated in descending ethanol row and subsequently stained either with haemtoxylin/eosin for recognizing various tissue types or Masson-Goldner trichrome for differentiation between collagen, muscle and bone tissue. Sandersons Rapid Bone Stain (SRS)/van Gieson (Dorn & Hart, Loxley, Al, USA) for differentiation of hard and soft tissue was performed directly after sectioning. SRS/van Gieson also known as Stevenol's blue stain/van Gieson, is a fast staining procedure on non-deplastified sections based on a mixture of methylene blue and potassium permanganate counterstained with van Gieson. Structures are stained as following: osteoid/soft tissue = blue/green, cell nuclei = blue/black and mineralized bone = yellow/orange.

3. Results

Similar to the laser microtome sections, the grinding preparations showed that the zirconia implant was completely surrounded by a layer of connective tissue (Fig. 3A + D). Magnifications up to 20 times revealed no detailed histological information because cell structures could not be clearly identified (Fig. 3B–C). However, when using laser microtome slices (10 μm), osteocytes were

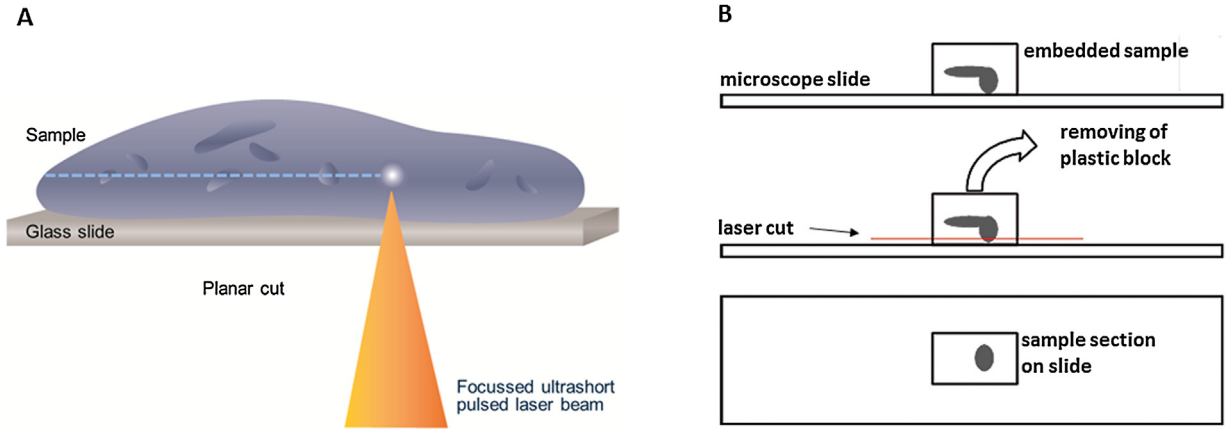

Fig. 2. Principle of optical cutting with an inverted system. A femtosecond laser is focused into the tissue sample. Cutting is performed within the laser focus by optical breakdown (A). The embedded sample glued to a microscope slide is cut by a laser, the bulk material of the sample is removed and the thin section remains on the slide (B).

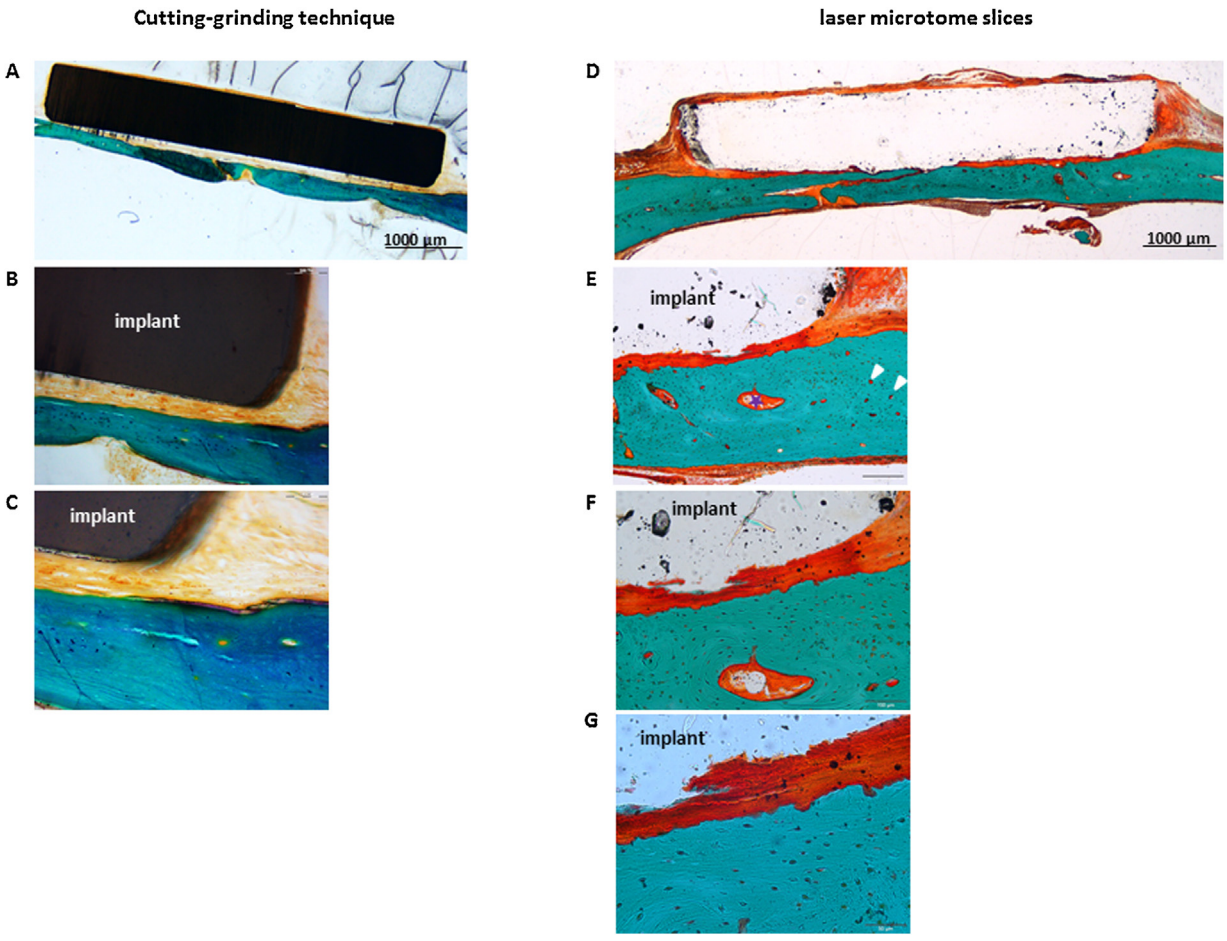

Fig. 3. Histological slices of zirconia implants stained with Masson–Goldner trichrome. (A–C) ground sectioning technology, (D–G) laser microtomy, (A + D) overview screen, (B + E) magnification: ×10, (C + F) magnification: ×20, (G) magnification: ×40; white arrows = osteocytes, violet star = resorption cavities.

already clearly visible in the bone tissue at a 10× magnification (Fig. 3E). Furthermore, osteons with Haversian canals and resorption cavities with primary bone marrow and blood vessels could be detected within the bones. Between the implant and the bone, a compact connective tissue layer devoid of visible blood vessels was present. The 40× magnification revealed that the collagen fibers were parallel arranged (Fig. 3G).

With the grinding technique, a minimum slice thickness of 50 µm could be achieved in case of PHB implants. At this thickness, the following details could be detected microscopically: the PHB implant had no direct contact to the bone and was completely embedded in collagen-containing tissue. It was, however, not possible, to reveal whether this tissue presented a capsule or connective tissue. As already described in the earlier publication (Gredes et al., 2015) bone regeneration could be found starting from marginal bone. Membranous bone is already present and kit lines were clearly visible (Fig. 4A–C). Both in the regenerated bone and in the lamellar bone osteocytes were detectable at a 20× magnification (Fig. 4B + C). Furthermore, it was possible to analyze the interface between implant and bone. In contrast to the grinding preparations when using laser microtome slices of the same samples, with a thickness of 10 µm, differentiation between capsule and connective tissue was clearly feasible. Even at low magnification, parallel collagen fibers were detected around the PHB implant, indicating a connective tissue capsule. Between these fibers and the bone loose connective tissue with blood vessels was present (Fig. 4E–K).

4. Discussion

This is the first study that shows the comparison of histological slices prepared by a cutting-grinding technique and laser microtomy. The use of laser microtomy to cut various hard tissues (e.g. bone, teeth), implants (e.g. stented vessels) as well as bone substitution materials (e.g. TCP scaffolds) has been demonstrated in some publications, although it has been claimed that ceramics cannot be cut (Lubatschowski et al., 2012; Richter et al., 2014; Will and Richter, 2015; Boyde, 2018). In addition, laser microtomy can also be used to cut native tissue, plants and wood (Johansson and Sandberg, 2007). The present study has shown that ceramic implants (zirconia implants) can be cut as well as PHB (polyester) by using the femtosecond laser technology. The direct comparison of bone-zirconium implant grinding preparations with laser microtome sections showed that more fine structures could be distinguished after using the laser microtome. Using sections produced by laser microtomy, tissue structures were clearly visible in the microscope even at 40× magnification, especially in the boundary layer between implant and bone. So far, this boundary layer was rather difficult to identify or was missing completely, if it came to the loss of the implant in the grinding process. Advantage of the laser microtome cuts is the lack of artifacts such as e.g. adhesive residues or sandpaper abrasion. These artifacts are clearly recognizable on the preparations of Mai et al. (2012), which were produced by means of cutting-grinding technique. The reason for this is that the slice thickness of the preparations differs by a factor of ten. In

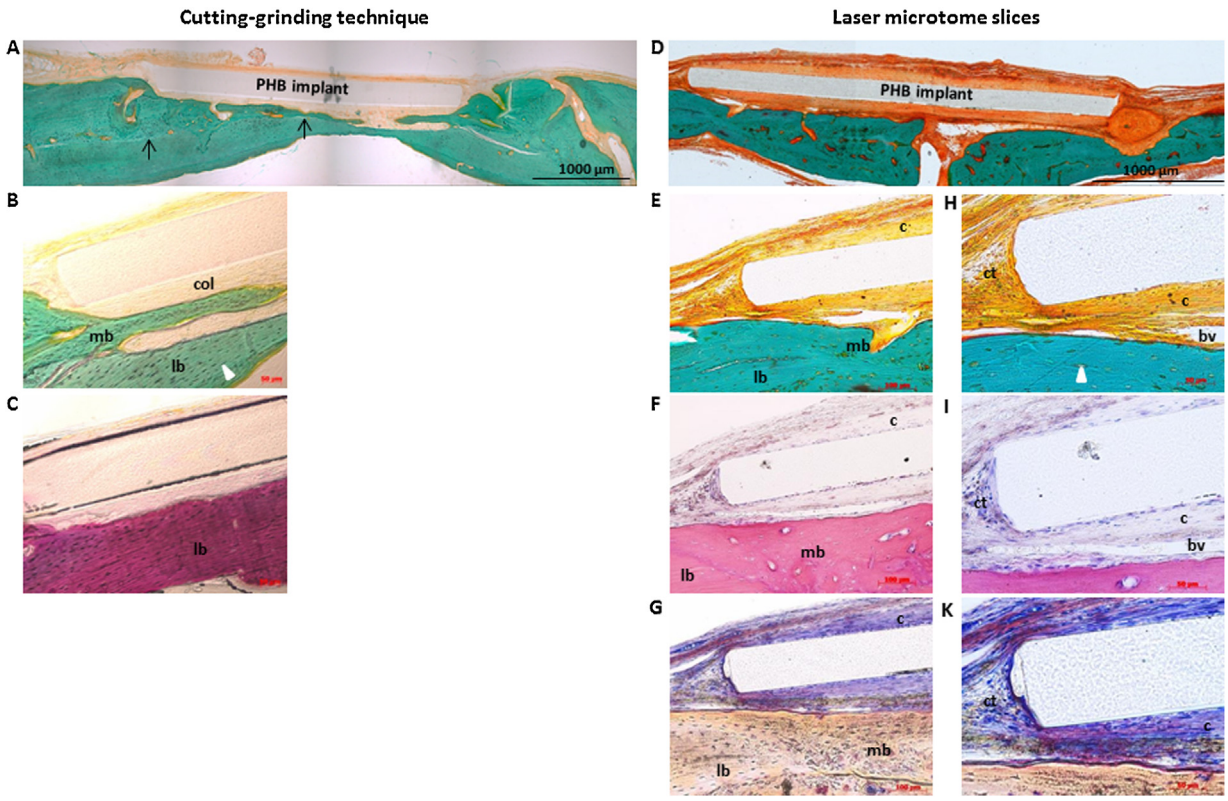

Fig. 4. Histological slices of PHB implants produced using ground sectioning technology (A–C) or laser microtomy (D–K) and stained with Masson–Goldner trichrome (A–B, D–E, H), hematoxylin/eosin (C, F, I) or Sandersons Rapid Bone Stain/van Gieson (G, K). Overview screen (A + D), magnification: ×20 (B–C + E–G), magnification: ×40 (H–K); ct = connective tissue, c = capsule, col = collagen fibers, mb = membranous bone, lb = lamellar bone, bv = blood vessel, white arrows = osteocytes, small black arrows = bone kit lines.

the case of zirconium implants, laser microtomy is clearly superior to the cutting-grinding technique.

In contrast, a significant advantage in the histological preparations containing PHB is not immediately apparent. PHB is a well-cuttable polyester that can also be sliced with conventional microtome blades (Gredes et al., 2015). However, the problem is on the one hand that the bone material has to be decalcified and on the other hand, the tissue wrinkles quickly when cutting. This is due to the different hardness of the materials used. Artifact free sectioning at the interface is essential because the interface between implant and tissue is the most important region of interest in the specimen. The laser microtomy avoids both, decalcification and artifacts. Smooth preparations are created, so that even at high magnifications fine structures can be reproduced sharply.

Regardless of the better visible histological structures, the advantage of laser microtomy over the cutting-grinding technique is above all the faster processing time of the samples. Thus, Will and Richter (2015) reported that in comparison to cutting-grinding technique small cuts are possible to be made 5× faster with the laser microtome in average. For large preparations, the entire production takes about 33 min instead of 123 min in case of the cutting-grinding technique. In addition, if using laser microtome, a significantly higher number of possible sections of a sample could be produced, whereby more specific investigations, especially in the field of immunohistochemistry are possible. It was described that between 3500 and 7000 slices per tissue block (35 × 35 mm or 14 × 14 mm) per technician per year can be prepared. 1/4–1/5 of them was possible by means of the cutting-grinding technique (Will and Richter, 2015). In the case of dental implants, the possibility that significantly more cuts can be made plays an essential role. For an implant diameter of 4 mm, a maximum of 2–3 sections can be prepared by the cutting-grinding technique with a complete longitudinal view of the implant (Gredes et al., 2014). It is known that this method leads to a sample loss of more than 0.5 mm per cut (Will and Richter, 2015; Boyde, 2018), whereas using laser microtomy causes no significant tissue loss between the cuts. Another disadvantage of the cutting-grinding technique is that, in contrast to the laser microtomy no serial cuts can be made. The laser microtome is suitable for production of serial slices of a thickness between 5–100 µm (Lubatschowski, 2007; Lubatschowski et al., 2012). By proper use, it is feasible to produce 5–15 µm thick tissue sections with the cutting-grinding technique but not in series due to the already mentioned tissue loss (Donath and Breuner, 1982; Rohrer and Schubert, 1992; Cano-Sanchez et al., 2005). Another problem is the frequent loss of the implant in the course of the grinding process.

Another important difference between laser microtomy and cutting-grinding technique is that laser microtomy does not cause thermal damage of the tissue due the use of a femtosecond laser. The need for water to cool the specimen during cutting and grinding is completely eliminated in case of laser microtomy. It is known that due to the short exposure time of 100–400 fs no thermal damage occurs outside the cutting zone. The short pulse durations allow only a very small amount of energy in the range of a few nanojoules per pulse, which is deposited in the sample (Lubatschowski, 2007; Lubatschowski et al., 2012). It has recently been shown that the structural integrity of mouse lungs cut by laser microtomy is comparable to that of micro-Ct images. Almost no local displacement could be established between resin-embedded laser microtome sections and the micro-Ct images. Furthermore, the displacement index was increased two tines if using paraffin-embedded samples cut with standard microtome in comparison with laser cut resin samples (Albers et al., 2018).

In conclusion, at the histological level, laser microtome sections are superior compared to thick sections (≥30 μm) prepared by the cutting-grinding technique. In addition, laser microtomy has the advantages of time savings and tissue-saving production of serial cuts.

Acknowledgement

We would like to thank Diana Jünger for the excellent technical assistance.

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