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RH 414 a New Dye to stain non - Decalcified Bone Tissue

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Introduction

The preparation of histological specimens in dentistry is hampered by the fact, that most of the samples are containing a combination of soft and hard tissues. If non-decalcified samples are prepared, the hardness of the human enamel or the material of endosseous implants does not permit to prepare serial sections using diamond knifes. In 1982 Donath and Breuner described the sawing and grinding technique to produce non-decalcified histological specimens for microscopic analysis.
This type of sample preparation has become a standard in periodontal as well as implantological research. One of the disadvantages concerning this technique is, that during preparation a considerable amount of tissue is lost and also the plane and direction in which the sample is sawn may not be changed. Thus only 2 - 4 histological samples may be obtained, if 1 implant with surrounding tissues is prepared.
This technique limits also tissue staining with monoclonal antibodies, which in most cases require the preparation of frozen specimens or the fixation in paraffin.
It is known from earlier investigations by Grötz et al. (1997, 1998), that confocal laser scanning microscopy (CLSM) may be used to analyze dental hard tissues and bone.

Objective

It was the aim of this study to test carbocyanine iodides (DiOC) and a propyl-butadienylpyridiniumbromide dye (RH 414) for their suitability in the analysis of non-decalcified bone tissues by CLSM.

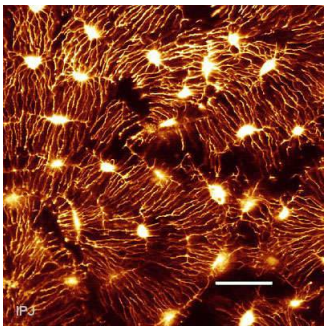


Fig. 1a: Cortical bone after RH 414-H2O staining and laromin C- embedding, CLSM. Bar = 30 µm

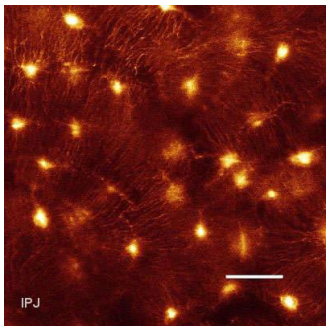


Fig. 1b: Same sample as in Fig. 1a 60 µm below the surface, CLSM. Bar = 30 µm

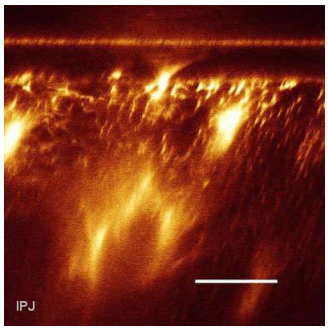
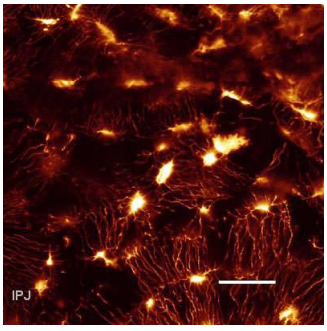
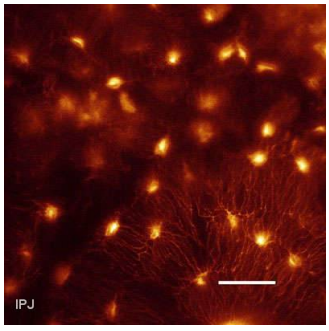


Fig. 1c: Same sample as in Figs.1a,b; optical cut in xz-direction, Bar = 30 µm





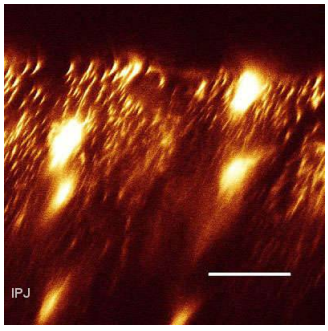
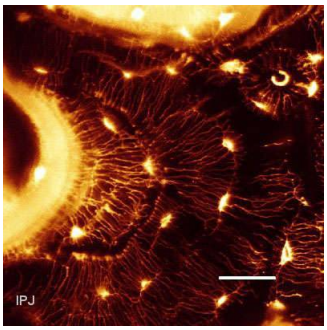
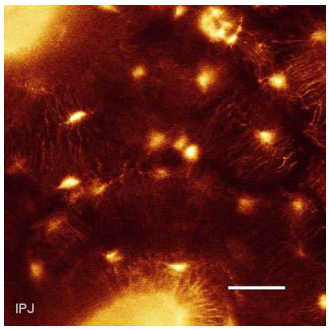


Fig. 2a: Cortical bone after RH 414-ethanol staining and paraffin- embedding, CLSM. Bar = 30 µm

Fig. 2b: Same sample as in Fig. 2a 60 µm below the surface, CLSM. Bar = 30 µm

Fig. 2c: Same sample as in Figs.2a,b; optical cut in xz-direction, Bar = 30 µm





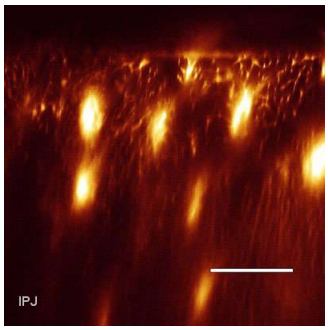


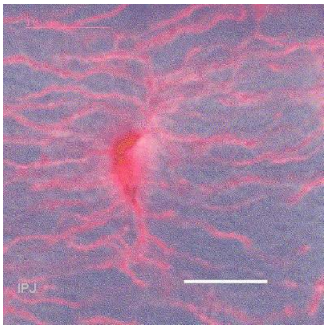
Fig. 3a: Cortical bone after RH 414-ethanol staining and laromin C - embedding, CLSM. Bar = 30 µm

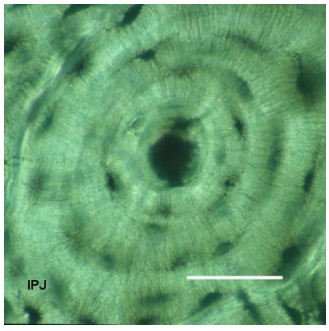
Fig. 3b: Same sample as in Fig. 3a 60 µm below the surface, CLSM. Bar = 30 µm

Fig. 3c: Same sample as in Figs. 3a,b; Optical cut in xz-direction, Bar = 30 µm

Material and Methods

Bone samples from the proximal tibia of 3 healthy dogs were taken in conjunction with major surgical procedures.
Immediately afterwards the specimens were fixed in 4% formaldehyde and kept in the refrigerator. 6 small block sections were prepared of approximately 4 x 4 mm size. 3,3' dihexyloxacarbocyanine iodide, 3,3' diheptyloxacarbocyanine iodide (DiOC 6 and 7) and N-(3-triethylammoniumpropyl)-4-(4-(4-(diethylamino)phenyl) butadienyl)pyridiniumbromide (RH 414) were tested for the first time concerning their staining properties in mammalian tissues. The different dyes were dissolved either in 1 ml double-distilled water or in 1 ml ethanol and the samples were incubated in the staining solution for 24 h.
Subsequently half of them were embedded in Laromin C 268 (BASF, Ludwigshafen, Germany) in combination with glycidether (Merck, Darmstadt, Germany). The other part was dehydrated in increasing concentrations of ethanol and embedded in paraffin (Merck, Darmstadt, Germany). Subsequently the tissues were fixed to microscopic slides.
All specimens were analyzed in the confocal laser scanning microscope (Leica, TCS 4 D, Wetzlar, Germany).After microscopic analysis one part of the samples was further processed according to the technique described by Donath et al. (1982), i.e. they were sawn and grinded. for conventional light microscopy. In these samples additional staining with Toluidin-blue and basic fuchsin (CPC, Paragon, New York, U.S.A.) was performed.The aim of the microscopic analysis was to identify cellular elements in the bone and to investigate the possibility of tomographic analysis.





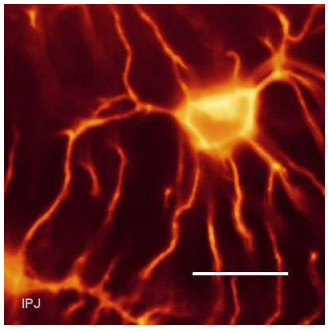


Fig. 4: Osteocyte (RH414), fluorescine and light microscopy (double overlay), CLSM, Bar= 10 µm

Fig. 5: Double staining of bone tissue sample (RH 414 and toluidine blue/fuchsin), light microscopy; Bar = 30 µm

Fig. 6: Single osteocyte with canaliculi, RH 414 staining and CLSM. Bar = 10 µm

Results

First experiments with DiOC6 and DiOC7 didn't result in adequate cellular fluorescence (results not shown). Instead RH 414 staining resulted in a strong signal of all cellular elements in the bone tissue (Figs. 1a, 2a, 3a). This was independent from the mode of dissolution of the dye and embedding of the tissue. It could be demonstrated, that cellular elements still were discernible in a depth of approximately 60 µm - 100 µm below the bone surface (Figs. 1b, 2b, 3b). This was further confirmed by optical scans in the xz-direction, which clearly demonstrated that even in a depth of approx. 100 µm cellular elements could be identified (Figs. 1c, 2c, 3c).
RH 414 staining of gingival tissue, lead to a rather uniform picture, demonstrating, that this dye reacts with the membranes of all cellular elements (results not shown).
The high resolution of the laser scanning microscope made it possible to even analyze single osteocytes demonstrating their canaliculi in the tissue matrix (Fig. 6).
RH 414 and toluidine/fuchsin double-staining is possible, thus membrane labelling does not hamper conventional microscopic analysis (Figs. 4 and 5).

Discussion and Conclusions

Our results demonstrate, that staining with RH 414 in combination with microscopic analysis by CLSM enables to analyze cellular elements of the bone tissue even 60 to 100 µm below the sample surface.
This is of considerable value, since with this technique non-decalcified bone may be analyzed in regions, undisturbed by sample preparation as already outlined by Grötz et al. (1998). Staining of bone tissue with RH 414 is an easy technique, and rather inexpensive as compared with monoclonal antibody labelling.
With this new technique it will be possible to analyze all hard tissues and get new insights in the number and distribution of cellular elements in a calcified matrix.

Acknowledgements
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Abstract

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References