

MicroCT-based imaging of microvasculature within the bone tissue

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Abstract

Angiogenesis is essential for skeletal development, bone healing and regeneration. Improved non-destructive, three-dimensional (3D) imaging of the vasculature within bone tissue would benefit a multitude of research areas, especially implantology and tissue engineering.

X-ray microtomography (microCT) is a well-suited non-destructive 3D imaging technique for bone morphology. For the detection of vessels, using contrast-enhanced microCT imaging is essential. Limited contrast between perfusion agents and mineralized bone has been the major drawback of this approach, making their distinct segmentation problematic. A decalcification step resolves this issue but inhibits simultaneous assessment of intracortical bone microstructure and vascular morphology. Moreover, the problem of contrasting becomes further compounded in samples with metal implants.

This study describes μ Angiofil-enhanced microCT-based visualization of vasculature within bone tissue in small and large animal models, with and without decalcification. We present simultaneous microvascular and bone imaging in murine tibia, murine bone metastatic model, pulp chamber, gingiva and periodontal ligaments. In a large animal model (minipig) we perform visualization and segmentation of different tissue types and vessels in the hemimandible containing metal implants.

Our manuscript introduces the first non-destructive approach for 3D imaging of the vasculature within soft and hard tissues in the vicinity of metal implants in a large animal model.

Introduction

Angiogenesis is an essential physiological vascularization process for skeletal development and growth as well as for bone healing and regeneration. This formation of new blood vessels from pre-existing vessels is crucial for successful bone formation and repair [5]. In addition to carrying nutrients and growth factors, those newly formed blood vessels are a delivery route of stem cells and progenitor cells to the bone-defect site [6,7,8]. The structural nature of skeletal tissue makes three-dimensional (3D) imaging of its vasculature extremely difficult. Histology, a destructive and two-dimensional approach, still remains a gold standard for assessing vasculature in bones [9]. Classic soft tissue imaging techniques such as light sheet microscopy or confocal laser scanning microscopy face challenges in their application due to the encasement of blood vessels in calcified tissue [9]. Despite recent advancements in tissue clearing-based imaging methods for craniofacial bones in a mouse model [10,11], the application of such methods remains challenging. In the case of bone grafts, many of the synthetic ones fail to bridge critically sized defects due to their inability to promote vascularization [4,12]. In essence, simultaneous non-destructive 3D imaging of the vasculature within bone tissue and of the bone tissue itself, especially in the case of larger bone grafts, has been a challenge for decades [4,13]. Thus, many research areas benefit from an improved three-dimensional (3D) imaging of the vasculature within bone tissue: for example bone metastatic disease, bone biology, tissue engineering, implantology, reconstructive surgery and healing of both small and critical size bone defects with or without bone grafts.

In the last decades, X-ray micro-computed tomography (microCT) gained recognition as a non-destructive 3D imaging technique for bone morphology [14]. Due to the inherently low difference in X-ray absorption levels between vessels and different soft tissues it is not easily feasible to distinguish such structures within the bone. To unambiguously detect vasculature within bone it is thus necessary to instill the vessels with either a contrast agent or use a casting method to otherwise fill the blood vessels. Currently existing protocols for imaging the vasculature within the bone via a vascular replica, have drawbacks like showing disjoint vascular components or completely missing vascular segments [9,15]. It was also reported that the contrast difference between the perfusion agent to generate the replica of the vascular network and the mineralized bone make it problematic to perform a distinct segmentation of the bone tissue and the vasculature. This issue can be overcome by decalcifying samples prior to vascular imaging. Such a decalcification procedure makes simultaneous assessment of the intracortical bone microstructure and the vascular morphology impossible, though [9].

Intravascular contrast-agent-enhanced microCT has the potential to overcome these issues. It has become a method of choice for the evaluation of angiogenesis in bone tissue engineering and remodeling applications [2,4,16]. Barium sulfate and Microfil have been applied as the two most common contrast agents in studies on the vascularization of bone tissue. Only some selected studies [2] managed to show better perfusion and thus visualization when perfusing the vasculature with barium sulfate. Previous studies have reported disadvantages associated with barium sulfate suspensions, including higher viscosity, which can sometimes result in incomplete vascular filling and weak or inhomogeneous signal, particularly in higher resolution scans [2]. These issues may be attributed to particle aggregation, as indicated by various studies [17,18,19].

Although Microfil has probably been applied in a larger number of vasculature imaging studies than barium sulfate, Microfil was reported to have disadvantages like poor or incomplete filling of the vasculature [2,18,19] or vascular damage [20].

The problem of contrasting the vasculature becomes even further compounded in the presence of a metal implant. Such implants have revolutionized the treatment of patients with missing teeth or injured joints and bones [21,22]. Interactions occurring at the implant-tissue interface are widely

believed to play a crucial role in the success of implant placement and healing [21,23]. MicroCT is the only available approach to non-destructively investigate an intact bone-implant interface in 3D [21].

Beyond studying the anatomy and physiology of angiogenesis and vasculature of the bone itself, the contribution of the vascular component in the healing process around bone-borne implants is also considered an area of study meriting further experimentation [4,24]. To date, only few studies tried to assess and visualize the vasculature surrounding implants using microCT imaging, for examples see [25], where Wang et al. assessed a region of 100 μm around the implant surface and [26], where Bhargava et al. present VascuViz, a pipeline for vascular systems biology and compare it to other vascular imaging workflows. Both these studies image the samples at approximately 7 μm voxel size, inhibiting the assessment of microvessels with a diameter of approximately 40 μm or less. To the best of our knowledge, no study has managed to resolve the microvascular component in a large animal model due to the technical limitations of the perfused contrast agent and the applied microCT imaging technique.

The present study introduces the polymer-based contrast agent μ Angiofil for high-resolution microCT-based visualization of microvasculature within bone tissue in small and large animal models, both with and without decalcification of the bone. Moreover, we show that μ Angiofil is suitable for simultaneous imaging and subsequent analysis of peri-implant hard and soft tissues as well as their vascularization in the vicinity of metal implants in a large animal model.

Materials, Methods and Results

Animals

In this study we used one transgenic VEGF male 21 months old mouse (see [27] for more details), five 6 weeks old CB17SCID male mice and two Göttingen minipigs. Animal procedures were performed in accordance with the applicable Swedish, Israeli or Swiss legislation on the protection of animals and were approved by the corresponding committees.

The murine experiment were approved by the local ethical committee of the Canton of Bern, Switzerland under the permit number BE 55/16. Minipig experiments were approved by the Malmö/Lund regional ethical committee, Sweden under license 5.8.18-15672/2019.

Contrast-enhanced microangioCT of mice using μAngiofil

The contrast agent μAngiofil (Fumedica AG, Switzerland) was prepared according to the manufacturer's recommendations. The perfusion of the mice was performed as previously described [28,29]. Briefly, heparinized animals were deeply anesthetized, the thorax and the peritoneal cavity were opened with scissors to expose the descending aorta. The exposed aorta was then cannulated in either antegrade (for the perfusion of the hind limbs) or retrograde direction (for the perfusion of the head and teeth) with a Venflon cannula (26 GA). Afterwards, the blood was flushed out with warm PBS solution. Several cuts of the liver edge allowed for effusion of the blood and buffer solution. Thereafter, the selected part of the body (hind limbs or head and teeth) was perfused with μAngiofil at 1-1.5 ml/min using a syringe pump. The perfusion lasted until the organ of interest appeared completely blue [29,30]. In bones, it is not possible to visually monitor this color change, thus perfusion of the neighboring soft tissues serves as an indirect marker of sufficient perfusion within the bone. To achieve correct perfusion of the vessels within the bone, we prolonged the perfusion time by instilling at least 2 ml of extra volume of contrast agent after all the superficial tissues of the extremity or head turned blue.. After harvesting, the samples were fixed by immersion in 4% paraformaldehyde (PFA) solution and then imaged using microCT scanners (Bruker microCT, Kontich, Belgium).

Figure 1 shows the bone microstructure and vascularization of a tibia of a 21-month-old VEGF transgenic male mouse. This visualization approach enabled a simultaneous display of bone, its vascularization, and their segmentation. As presented in our other study, we were able to quantitatively show that the age-related osteoporosis diminishes with improved vascularization [27].



Figure 1: microangiCT of proximal murine tibia of a 21-month-old VEGF transgenic male mouse. After perfusion with μ Angiofil the murine tibia was harvested, fixated in 4% PFA and imaged by microCT. Arrowheads mark microvessels within the tibia. The diameter of the tibia shaft is around 1 mm. On the right side of the image one can distinguish the bigger epiphyseal vessels. The bone tissue appears white at the plane of the virtual section through the microCT-dataset. Scanning parameters (SKYSCAN 1172): acceleration voltage 50 kV, current 200 μ A, 2.98 μ m voxel size, 360 degrees scan, 0.05° rotation step, frame averaging 3.

Decalcification of the μ Angiofil-perfused murine tibia

It is practically the standard in the field to decalcify the bone samples to enable the proper visualization and segmentation of vasculature within bone tissue [29]. We successfully established a bone-decalcifying protocol (for murine hind limb) with 10% Ethylenediaminetetraacetic acid (EDTA) solution [31] by adapting previous work [15]. Decalcification of bone tissue does not negatively influence the bone structure itself, but reduces the X-ray absorption of bone tissue and thus makes it impossible to visualize simultaneously with the vasculature (Fig. 2). On the other hand, it enables tomographic imaging of the murine hind limb vasculature with less artifacts around the bone at lower acceleration voltages, which in most instances leads to shorter scanning times. Furthermore, segmentation of the vasculature in the tomographic datasets becomes easier and a subsequent histological evaluation of the sample is facilitated [31].

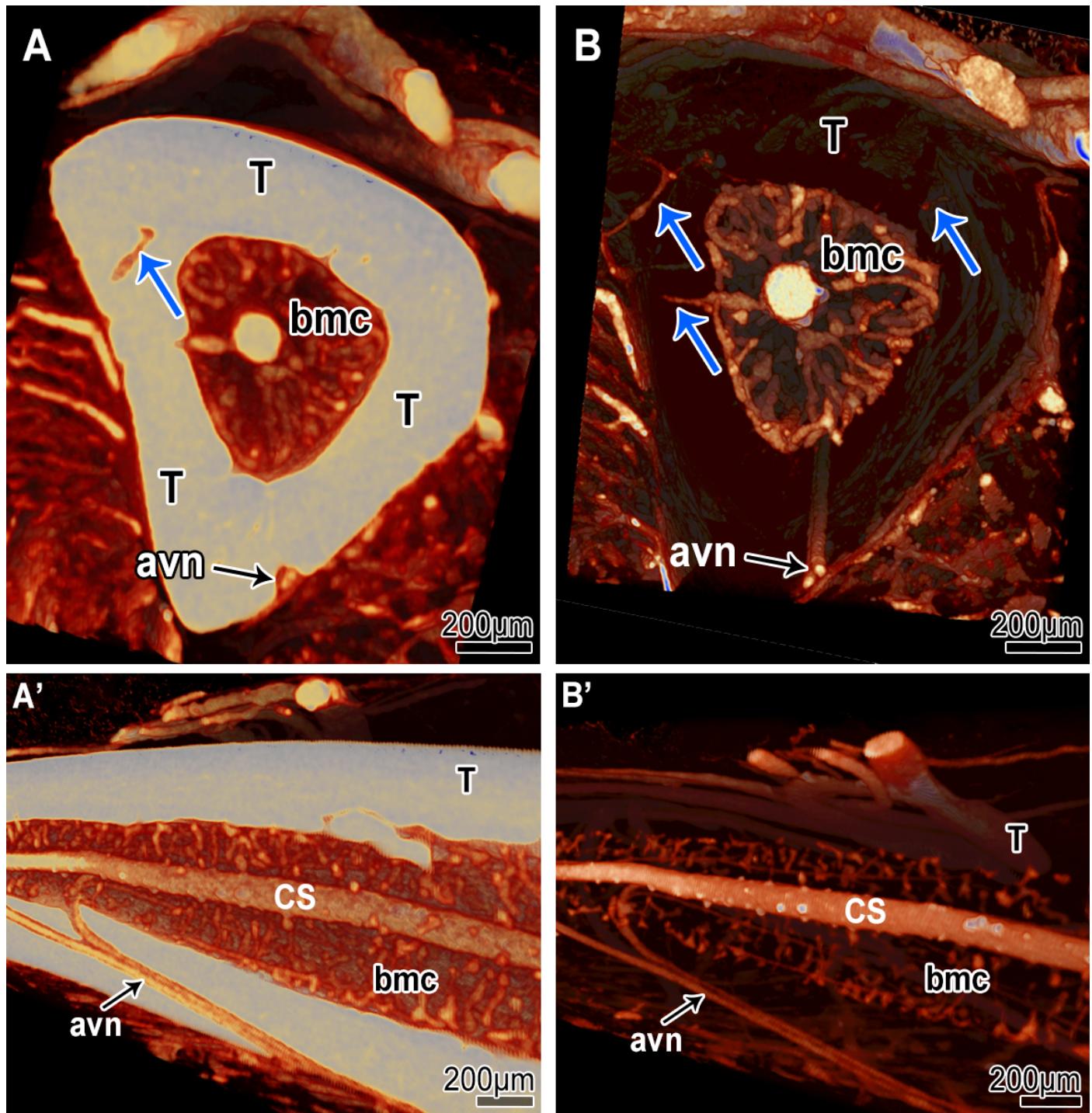


Figure 2: microangioCT-based visualization of the diaphysis of murine tibia before (A & A') and after decalcification with 10% EDTA (B & B'). In A and A' the tibia bone appears brighter and opaque due to higher X-ray absorption. In B and B' the tibia bone appears transparent due to its lowered X-ray absorption after decalcification. Due to the decalcification, connecting vessels between the periosteal vessels and the vessels of the bone marrow cavity (bmc) are more easily detectable (blue arrows in A vs. B). The visualization of the vessels within the medullar cavity (*central sinus* (CS)) is also improved. At the external surface of the tibia, supplying arteries are visible (*arteria et vena nutricia* (avn)). Scanning parameters (SKYSCAN 1172): acceleration voltage 59 kV, current 167 µA, 0.2 mm aluminum filter, 3.19 µm voxel size, 180 degrees scan, 0.1° rotation step, frame averaging 2.

Bone metastatic disease model, microangioCT

Assessment and 3D imaging of tumor vasculature is a challenging task, in almost every tumor model [32]. Due to the intraosseous location of tumor lesions, 3D microvascular imaging is even more challenging in a bone metastatic disease model. Since changes in bone and vasculature are believed to be of crucial importance for the progression of bone metastatic disease, simultaneous imaging of

bone and vasculature is crucial. The desire to simultaneously assess existing bone defects makes the aforementioned decalcification procedure highly unfavorable.

To verify the suitability of our microangioCT approach for this kind of experimental imaging we used the following murine bone metastatic disease model. 50000 PC3-M-Pro4Luc2 dTomato cells were injected into the tibia of 6 weeks old CB17SCID male mice as previously described [33,34]. The X-ray assessment (25 kV, 6 sec, Faxitron Bioptics, Tucson, Arizona, US) was conducted at days 7, 14, 21 and 28 after implantation to monitor the progression of the lesions. Prior to terminal anesthesia and perfusion with μ Angiofil as described above, the hind limb of interest was X-rayed using Faxitron Bioptics as a standard follow-up in this model (see insert in Fig. 3, Panel A). The harvested and fixated murine hind limb was then imaged using a desktop microCT scanner SKYSCAN 1272 (Fig. 3). The obtained dataset was reconstructed using NRecon Software v.1.7.4.2 (microCT Bruker, Kontich, Belgium) and visualized using CTvox Software v.3.3.1 (microCT Bruker, Kontich, Belgium). The visualization unambiguously displays intratumoral vasculature and extensive defects in mineralized bone tissue. Neighboring structures, such as the growth plate and the epiphysis or the menisci are also easily assessed (Fig. 3).

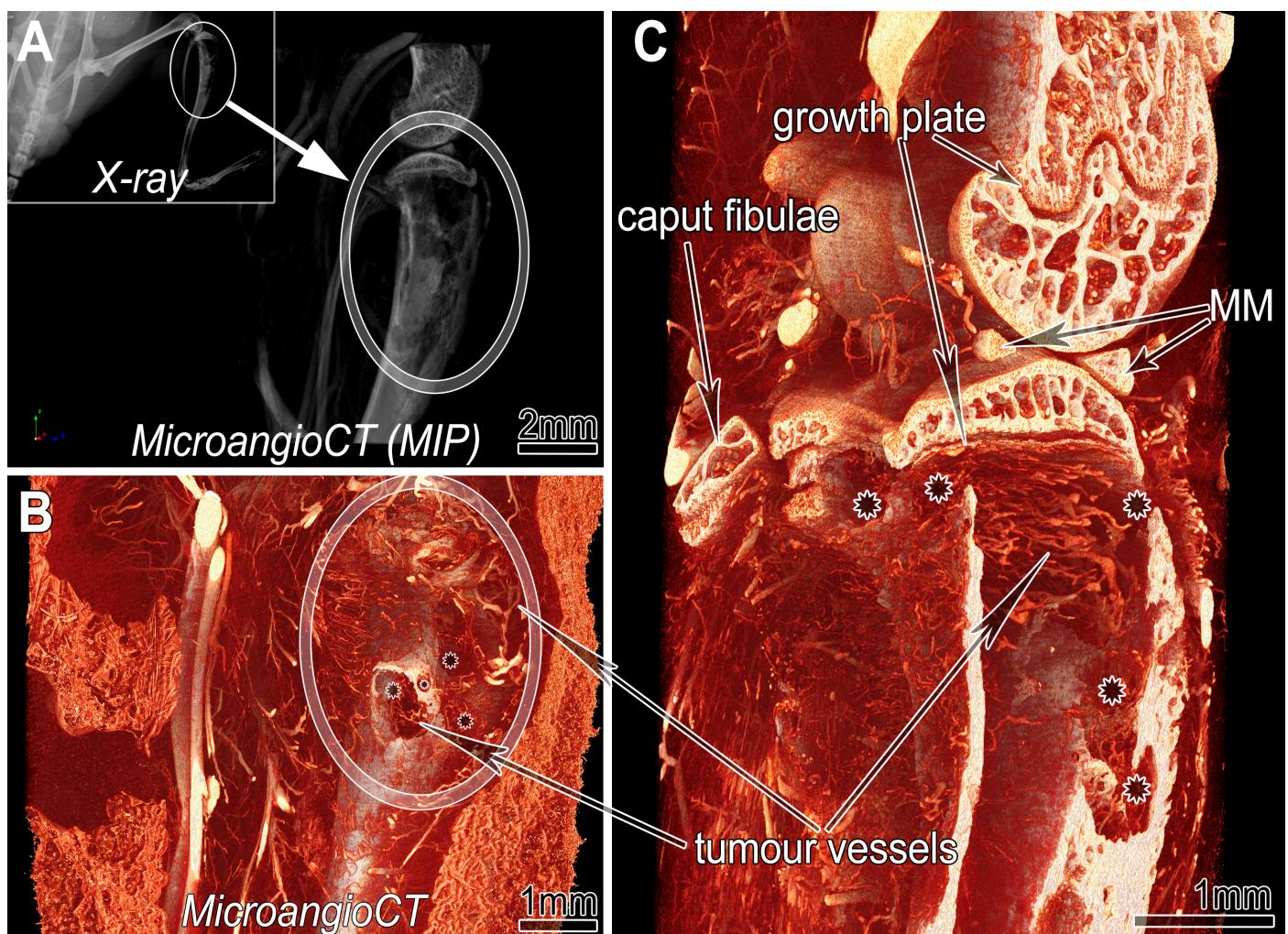
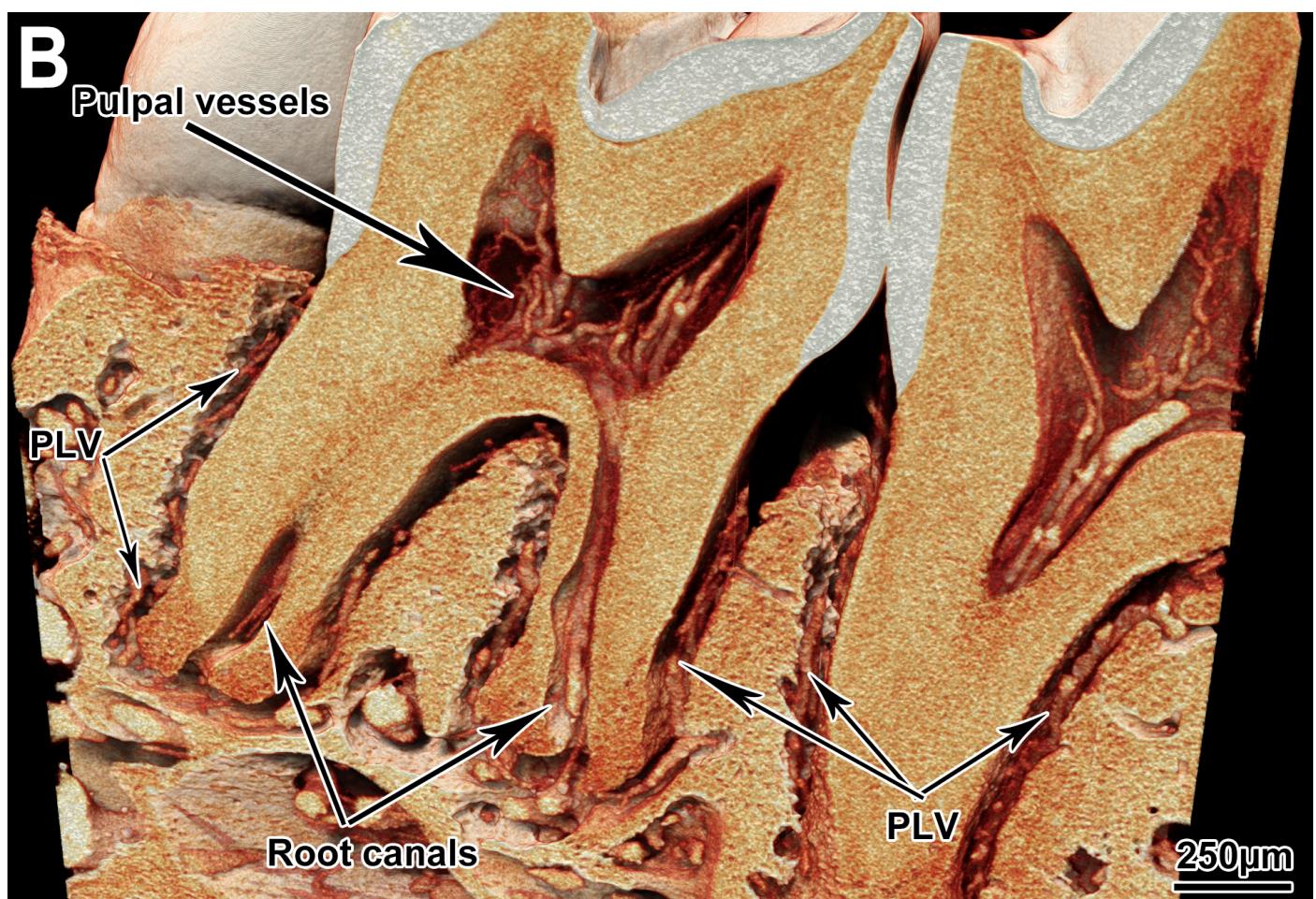
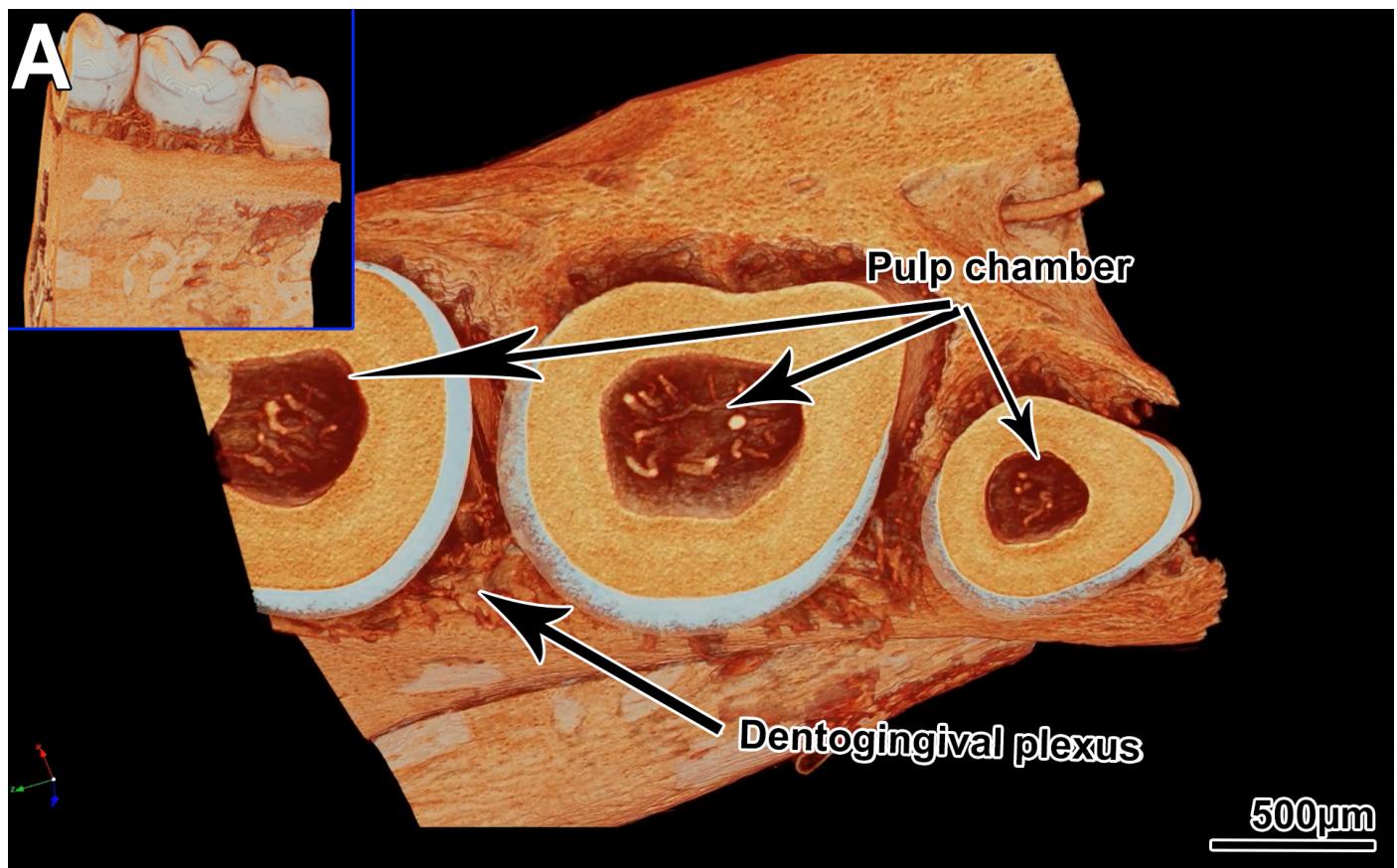


Figure 3: microangioCT of a xenograft tumor implanted into murine tibia. A: Maximum intensity projection (MIP) of the three-dimensional dataset of the investigated hind limb segment indicated in the inserted X-ray image of the mouse prior harvesting. Inset: X-ray image prior to terminal anesthesia. B: Virtual section through the microangioCT dataset visualizing remarkable defects represented as holes (asterisks in B and C) in the tibial bone at the tumor site (encircled). C: A more deeply positioned virtual section displaying the inner surface of the diseased tibia. Besides irregularly patterned tumor vessels (in B & C) further bony structures like growth plate or calcified parts of the medial meniscus (MM) are clearly distinguishable. Scanning parameters (SKYSCAN 1272): acceleration voltage 60 kV, current 166 μ A, 0.2 mm aluminum filter, 1.65 μ m voxel size, 360 degrees scan, 0.1° rotation step, frame averaging 3.

microangioCT of murine mandible/teeth

Murine mandibles and teeth are challenging samples due to the location of most of the vessels within the bone canals or in the proximity of the hard tissue and, correspondingly, the lack of larger bone-free volumes in which the vessels are easily distinguishable. Nonetheless, with the improved perfusion protocol (see above) and a well visualized difference between the X-ray absorption levels of μ Angiofil and mineralized bone tissue, we achieved appropriate imaging of the sample. The microvasculature of murine mandible, periodontal ligament, and the teeth (even within their pulp chamber) can be clearly visualized without undergoing a decalcification procedure (Fig. 4).



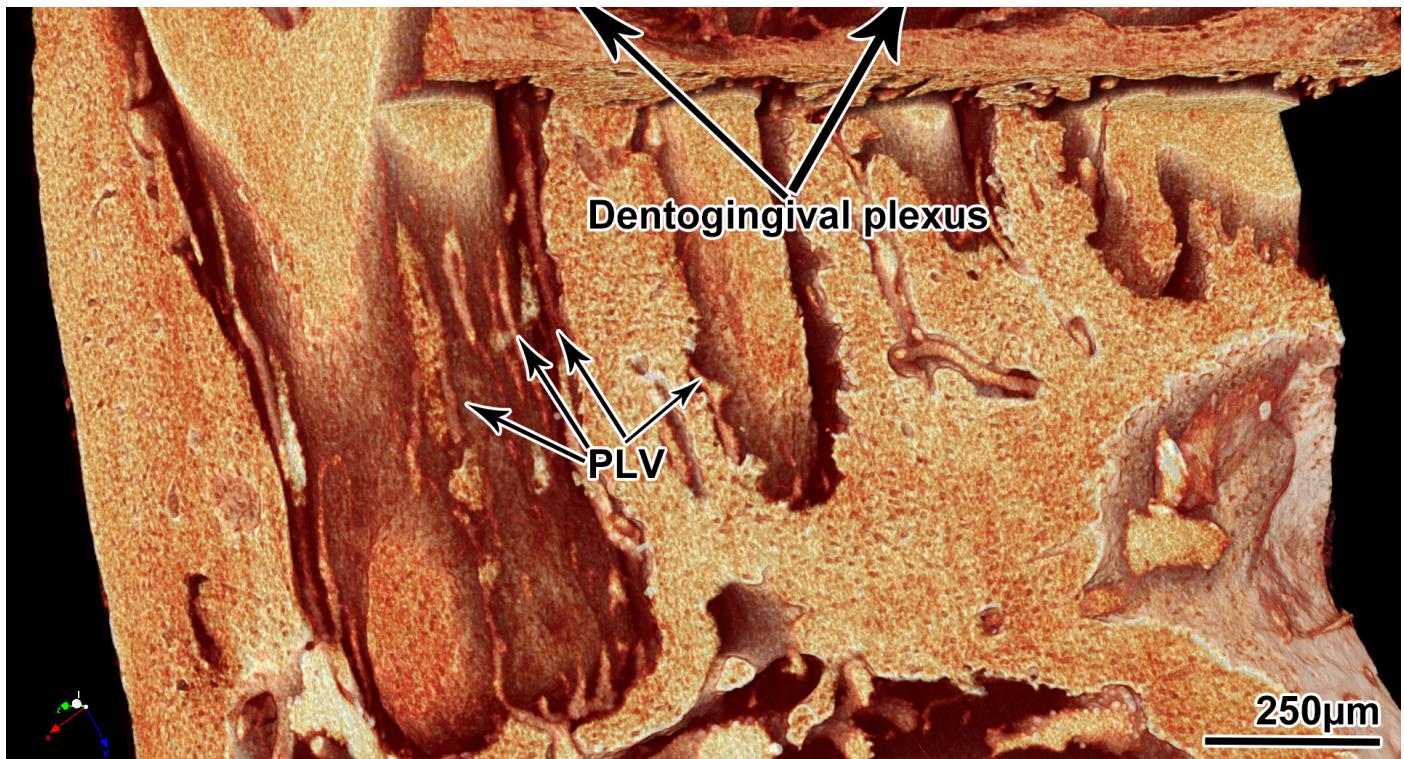


Figure 4: microangioCT of the vasculature of murine teeth. A: View onto the virtual horizontal section through the crowns of the murine teeth: The pulp chambers are visible, and the pulpal vessels are presented. Overview of the dataset in the inset at the upper left corner. B: Sagittal section through the mandible. The microvessels within the pulp cavities and root canals are distinct. C: A closer view onto the dentogingival plexus and periodontal ligament vessels (PLV). Scanning parameters (SKYSCAN 1172): accelerating voltage 80 kV, voxel size 1.0 μm , 0.2 mm aluminum filter, rotation step 0.1°, 360 degrees scan, frame averaging 4.

microangioCT of mandible in a large animal model (Göttingen Minipig)

Due to their similarity to humans in terms of anatomy, bone structure and bone remodeling, the Göttingen Minipig has become a well-accepted large animal model in preclinical dental and orofacial research [35,36,37]. The stricter legal requirements regarding primates and the societal resistance against the use of companion animals, such as dogs and cats, have further enhanced the usage of Göttingen minipigs as animal models [36].

Göttingen minipigs (Ellegaard Göttingen Minipig, Dalmose, Denmark) were intramuscularly anesthetized (25-35 mg/kg, Dexdomitor; Orion Pharma Animal Health and 50-70 mg/kg, Zoletil 100 Vet, Virbac) and heparinized with 300 IE/kg intravenously (Heparin LEO, LEO Pharma). After heparin infusion, the pigs were euthanized with an intravenous dose (100 mg/kg) of pentobarbital (Euthanimal vet, VM Pharma). *A. carotis externa* was accessed by blunt dissection through tissue of the ventral neck and cannulated (BD Venflon, 17G). After washing out the blood with PBS, the corresponding head side was selectively perfused with μ Angiofil through the arterial tree. The success of the perfusion has been visually controlled equivalent to the previously described perfusion in mice. After polymerization of μ Angiofil (~30 min), the mandible was excised and fixated in 4% PFA solution. They were later scanned using SKYSCAN 2214 or SKYSCAN 1273, and the datasets reconstructed and visualized using NRecon v.1.7.4.2 and CTvox Software v.3.3.1, correspondingly (both microCT Bruker, Kontich, Belgium). Our approach provides excellent imaging of the whole mandible vasculature down to the vessels within the root canals and pulp chamber (Fig. 5).

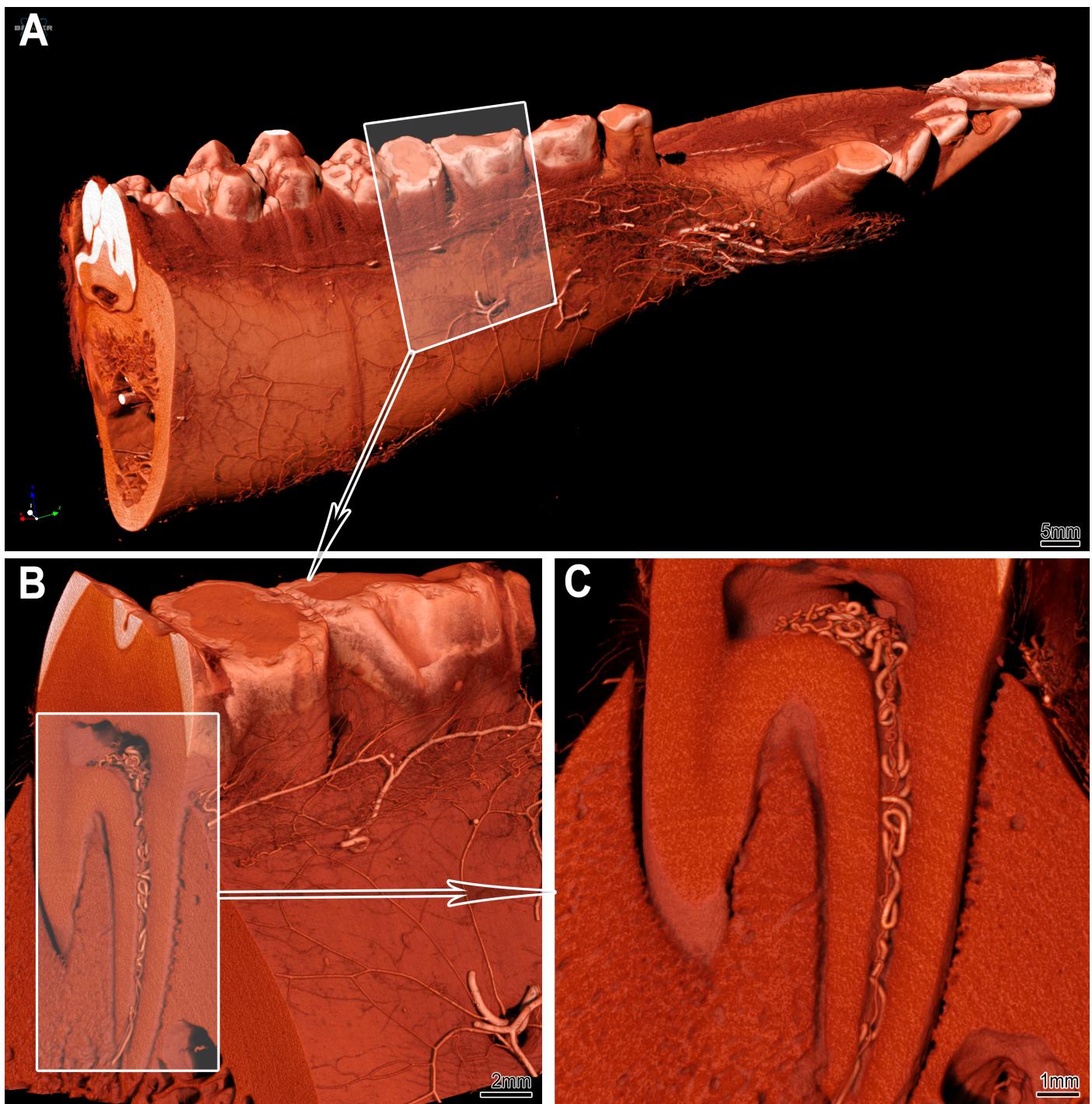


Figure 5: microangioCT of the minipig mandibula. Panel A displays the visualization of a right minipig hemimandible: the vasculature at the bone surface is clearly visible. The framed area in A marks the subvolume represented in panel B at higher magnification. Panel C displays the transverse section marked in panel B: the pulp chamber and root canal with the corresponding vessels are unambiguously visualized. Scanning parameters for images in panel A (SKYSCAN 1273) accelerating voltage 100 kV, current 80 μ A, 9 μ m voxel size, 1 mm aluminum and 0.2 mm copper filter, rotation step 0.15°, 360 degrees scan, frame averaging of 5. Scanning parameters for images in panels B and C (SKYSCAN 2214): accelerating voltage 100 kV, current 100 μ A, 8 μ m voxel size, 1 mm copper filter, 0.18° rotation step, 360 degrees scan, frame averaging 4. Due to the voxel size of 8-9 μ m, microvessels with diameter of 40 μ m or less cannot be visualized in such large samples.

As already mentioned, the Göttingen Minipig has become a very popular large animal model in dental research and is often used also for implantology studies. After researchers in the field realized that osseointegration is influenced by angiogenesis, there is understanding that angiogenesis and, correspondingly, vascular supply of the peri-implant tissue should be assessed as thoroughly as possible and preferably in 3D. So far, the only reliable approach to assess the vascular supply remains histology, which is limited to single two-dimensional sections. Based on the findings in those single histological sections, researchers try to assess the vascularization of the peri-implant bone. Even for

the microCT approach, it is a challenging task due to the presence of metal parts and, therefore, multiple objects with high density within the samples. The decalcification step could reduce the density of the sample but would inevitably lead to the loss of information on the bone microstructure making the simultaneous assessment of the bone and vessels impossible [9]. Another obstacle is to have a contrast agent that can be distinctly separated from both the metal implants and the mineralized tissue. With μ Angiofil it is easily possible to visualize and distinguish between soft tissue, bone tissue, contrast agent-filled vessels as well as metal implants according to their gray values in the histogram (Fig. 6, Panel C). Such imaging does not require bone decalcification and enables a straightforward segmentation of features of interest without cumbersome post-processing (Fig. 6, Panel D).

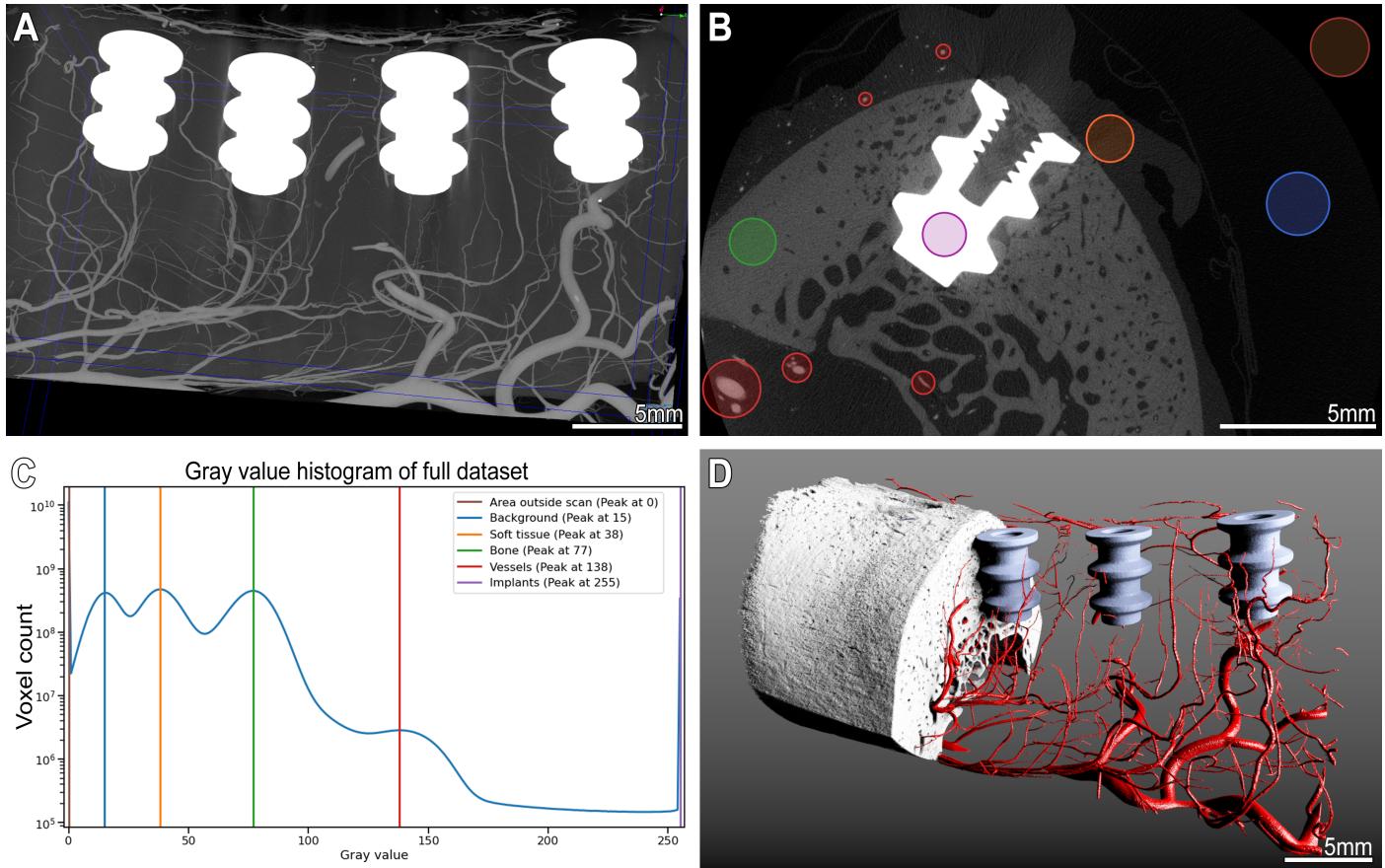


Figure 6: microangioCT of the peri-implant vasculature of a minipig mandibula. Panel A displays a MIP image of the minipig mandible with 4 metal implants and μ Angiofil-perfused vessels. Panel B: virtual transversal section through an implant within the mandible. Colored circles mark structures with distinguishably different grey values. See the histogram distribution and corresponding peaks with colors legend in panel C. Such differences in grey levels allow a straightforward segmentation of the structures of interest as displayed in panel D. Scanning parameters (SKYSCAN 2214): accelerating voltage 100 kV, voxel size 8.0 μ m, 1 mm copper filter, rotation step 0.18°, 360 degrees scan, frame averaging 4.

Discussion

There is an understanding in the bone biology, tissue engineering and related research fields, that adequate vascularization is a prerequisite for successful bone formation and regeneration as well as osseointegration of biomaterials [1,38,39,40,41]. Without high-resolution imaging, the interplay between vasculature and bone tissue cannot be adequately studied, making it impossible to influence or even control these processes. During the last decades, microCT gained recognition as a non-destructive 3D imaging technique for bone morphology [14]. It is considered the only available approach for 3D imaging of an intact bone-implant interface [21]. Unfortunately, the microCT-based distinction of the vessels from other soft tissue structures is not possible without intravascular casting or instillation of contrast agents. Moreover, although enabling the visualization of the vasculature, most such casting or contrast agents are difficult to distinguish from the non-demineralized bone tissue due to the lack of remarkable differences in their X-ray absorption levels [16]. For example, in case with Microfil, the mentioned lack of contrast requires decalcification of the samples before the bone vasculature can be properly visualized with a microCT scanner [9,16]. Some studies have described that barium sulfate suspension could be used for direct simultaneous imaging of vasculature and bone [15,42]. Many studies report higher viscosity of this agent in comparison to Microfil or μ Angiofil, though which leads to inconsistent vascular filling or even the inability to perfuse fine capillaries [19,42]. The hereby described approach with μ Angiofil provides more complete vascular filling [19,28] and is suitable for the visualization of the microvasculature within bone tissue with or without decalcification (Fig. 2). If a subsequent histological evaluation is desired, the developed decalcifying protocols enable correlative imaging of the same sample with preserved intravascular contrast agent [31].

Distinct differences in the attenuation between the μ Angiofil and bone tissue allow for assessing vascularization and bone growth without the need to make two scans (before and after decalcification) and register them, saving labor, scanning time and without damaging the sample composition.

The microangioCT approach we present here can be used for simultaneous visualization of the hard (and eventually soft) tissues and their vascularization in small and large animal models. One of the noteworthy possible applications of this approach is simultaneous visualization of the bone microarchitecture and microvasculature of the bone metastases in the murine xenograft tumor model (Fig. 3). Using such an approach, the response to specific treatments, including bone-targeted and antiangiogenic therapies, can be assessed within the same sample and in 3D, followed by histological examination if needed [43]. The presented method could offer additional insights into the interplay between angiogenesis and bone growth, bone lysis, and bone turnover, which have not been fully elucidated yet. The results will be crucial for selecting potential drug candidates and proper treatment decision and efficacy prediction. The decalcification step would be very disadvantageous for studying bone metastatic models. Such a step would hinder the thorough assessment of pathological processes in bone tissue. These processes may lead to so-called skeletal-related-events associated with shortened survival and deterioration of quality of life [44], and should therefore be avoided.

In dental research, preclinical models can be divided into small and large animals. Small animal models, particularly mice and rats, are highly popular, due to their practical size and cost-effectiveness. Small animal models, such as mice and rats, are highly popular due to their practical size and reduced cost. Moreover, there is a variety of genetically modified lines available which could facilitate imaging if using corresponding imaging modalities [21]. The microCT scan of correspondingly small samples perfused with μ Angiofil can be performed with a voxel size around 1 μ m, providing excellent detail resolution (Fig. 4).

Nonetheless, the small size of hemimandible samples and availability of the transgenic lines would allow for a recently published imaging approach [21] in the murine models. This tissue-clearing-based approach could be a viable option for the visualization of the peri-implant tissues and vasculature in small animal models. There are limitations of such a tissue-clearing-based imaging approach, namely i) differential shrinkage among soft and hard tissues, leading to anisotropic distortion in samples where both tissue types (plus eventual metal implant) are present; ii) limited depth (around 800 µm in a mouse model [21]) due to the challenges of achieving complete transparency of bone tissue. These limitations become even more critical in a big animal model with metal implants. Large anisotropic distortion of the sample may lead to the alteration of the common site of interest, namely the implant-tissue interface. Furthermore, the limitation of the visible depth makes such an approach impracticable for usage in large animal models.

The microangioCT approach does not have such limitations and can be easily applied for visualization of hemimandible and its vascularization in a large animal model like Göttingen minipig (Fig. 5). μ Angiofil has noticeably different attenuation than mineralized bone and it is useful when scanning the samples, which contain high-density materials, like metals. It is thus possible to visualize and distinguish soft tissue, bone tissue, vessels filled with the contrast agent as well as metal implants according to their gray values in the histogram (Fig. 6).

To the best of our knowledge, this is the first study demonstrating non-destructive 3D imaging of the microvasculature of bone in the proximity of metal objects/implants in a large animal model.

Limitations of the approach

The most significant limitation of the suggested approach is the general limitation of microCT imaging, namely, the correlation between the resolution and the sample size. If the sample is only a few millimeters in diameter, the voxel size may be as small as 1 µm or less enabling visualization of the microvasculature down to the capillary bed [28,29,30,31]. In case of large samples, like minipig hemimandible, the achievable voxel size is around 10 times larger (≥ 8 µm), and it is therefore, not possible to reach that level of resolution. In such samples the finest microvessels with diameter under 40 µm cannot be accurately visualized (see Fig. 5 & 6).

Nonetheless, the presented approach is, according to our knowledge, the best what can be achieved with the modern microCT scanners and is a big step forward for qualitative and quantitative imaging in tissue engineering and implantology research as well as many other related research fields.

Author Contributions

[Contributor Roles Taxonomy](#), as defined by the [National Information Standards Organization](#).

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Conflicts of interest

Author	Conflict
Benjamin Bellon	Employed by a company producing dental implants
Benjamin E. Pippenger	Employed by a company producing dental implants

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Supplementary Materials

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1. **Long-term cell-mediated protein release from calcium phosphate ceramics**
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