Simultaneous 3D Visualization and Quantification of Murine Bone and Bone Vasculature Using Micro-Computed Tomography and Vascular Replica

PHILIPP SCHNEIDER,1 THOMAS KRUCKER,2 ERIC MEYER,3 ALEXANDRA ULMANN-SCHULER,3 BRUNO WEBER,4 MARCO STAMPANONI,5,6 AND RALPH MÜLLER1*

1Institute for Biomechanics, ETH Zurich, Zurich, Switzerland
2Global Imaging Group, Novartis Institutes for BioMedical Research, Cambridge, Massachusetts
3Institute of Zoology, University of Zurich, Zurich, Switzerland
4Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland
5Swiss Light Source (SLS), Paul Scherrer Institut (PSI), Villigen, Switzerland
6Institute for Biomedical Engineering, ETH and University of Zurich, Zurich, Switzerland

KEY WORDS mouse; polyurethane; vascular corrosion cast (VCC); vascular contrast perfusion (VCP); barium sulfate; synchrotron radiation; morphometry

ABSTRACT Recent evidence suggests a close functional relationship between osteogenesis and angiogenesis as well as between bone remodeling and bone vascularization. Consequently, there is a need for visual inspection and quantitative analysis of the bone vasculature. We therefore adapted and implemented two different vascular corrosion casting (VCC) protocols using a polyurethane-based casting resin in mice for a true three-dimensional (3D), direct, and simultaneous measurement of bone tissue and vascular morphology by micro-computed tomography (μCT). For assessment of vascular replicas at the level of capillaries, a vascular contrast perfusion (VCP) protocol was devised using a contrast modality based on a barium sulfate suspension in conjunction with synchrotron radiation (SR) μCT. The vascular morphology quantified using the VCP protocol was compared quantitatively with the results of a previously established method, where the vascular network of cortical bone was derived indirectly from cortical porosity. The presented VCC and VCP protocols have the potential of serving as a valuable method for concomitant 3D quantitative morphometry of the bone tissue and its vasculature. Microsc. Res. Tech. 72:690–701, 2009.

INTRODUCTION

Growing evidence suggests that vascular architecture within bone- and vascular-derived factors play an important role regulating bone cell physiology and the concerted action of the bone cell network (Collin-Osdoby, 1994). In general, bone cell survival depends on the proximity to vessels providing nutrients (Ham, 1952). Moreover, interruption of the blood supply can lead to avascular necrosis (Crues, 1986; Glimcher and Kenzora, 1979). In particular, there are many indications for a close relationship between osteogenesis and angiogenesis as well as between bone remodeling and bone vascularization (Colnot, 2005). Recently, the coupling observed between bone resorption and bone formation during bone remodeling was proposed to be mediated by bone vascularization (Parfitt, 2000). Furthermore, vascular endothelial growth factor (VEGF) and angiopoietins were located at modeling and remodeling sites of growing bones of human neonatal ribs (Horner et al., 2001), and linear relationships between bone formation and vascular parameters in rat trabecular bone were identified (Barou et al., 2002). It is therefore essential to better understand the anatomical and functional relationship between bone tissue and the associated vascularization. However, simultaneous, direct and real three-dimensional (3D) methods for the quantitative assessment of both bone and vasculature are rare, especially in small rodents such as mice (Duvall et al., 2004). The lack of spatial resolution of current methods typically does not give access to the capillary and cellular level of the vascularization and the bone tissue. Consequently, there is a fundamental need for visual inspection and direct quantitative 3D analysis of murine bone and bone vascularization concurrently.

In general, vascular corrosion casting (VCC) in conjunction with scanning electron microscopy (SEM) has been widely used for imaging organ and tissue vasculature of many different species (Lametschwandtner et al., 1990). Casts are produced by filling an internal luminal system or space with a liquid medium that solidifies in situ. The surrounding tissue and bone is then removed (corroded) and the resulting replica is dried,
rendered conductive, and examined using SEM (Hodde and Nowell, 1980; Northover et al., 1980). In particular, VCC combined with SEM was applied for the study of the vasculature and the microvasculature of bones primarily in rats (Aharinejad et al., 1995; Hirano et al., 1996; Morini et al., 1999, 2006; Okada et al., 2002; Pan- narale et al., 1997; Stanka et al., 1991). While vascular corrosion casts represent the 3D architecture of the vascular network, SEM can only provide two-dimensional (2D) data. Because of these limitations, photogrammetric methods have been devised, where stereoscopic SEM images are digitally acquired (Komatsu et al., 1999; Malkusch et al., 1995; Manelli et al., 2007; Minnich et al., 1999;). Nevertheless, these methods exclusively allow the reconstruction of the vascular surfaces in terms of height maps and hence, are inherently pseudo-3D only (Manelli et al., 2007). Moreover, there is a wide range of error possibilities in the field of SEM photogrammetry that has to be taken into account during image acquisition and through image quantification (Minnich et al., 1999), which additionally complicate accurate morphometric measurements.

For a true 3D and direct assessment of the vasculature, X-ray micro-computed tomography (µCT) and synchrotron (SR)-based µCT techniques have been adapted (Heinzer et al., 2006; Jørgensen et al., 1998; Marxen et al., 2004; Flouraboue et al., 2004; Toyota et al., 2002) and were used in combination with VCC and with a technique we would like to call vascular contrast perfusion (VCP). For VCP, the vasculature is perfused with a highly X-ray absorbing contrast agent, and unlike VCC, the surrounding tissue is not always corroded for CT imaging. Both vascular replica techniques (VCC and VCP) were primarily applied in rodents. Recently, µCT along with VCP was used without corrosion in rats to study weight-bearing induced vessel formation during distraction osteogenesis (Moore et al., 2003) and in the mouse to analyze collateral vessel development after ischemic injury (Duvall et al., 2004). However, quantitative evaluation of bone vascularization in small rodents like mice remains a challenging task due to problems of casting material, specimen preparation as well as tomographic imaging. Recently, a new polyurethane (PU)-based casting material was described (Krucker et al., 2004; Meyer et al., 2007) with improved chemical and physical characteristics that allow less fragile reproduction of vasculature, increasing X-ray opacity, and high reproduction quality. Here, we propose an integrative approach for the simultaneous assessment of murine bone and its vasculature. This included the use of the PU-based casting material with three different adjusted vascular replica protocols (two VCC, one VCP) to optimize X-ray opacity, partial tissue maceration to keep bone intact, and the necessary sample stability for conventional and SR µCT. This allowed application of CT to image vascular replicas and bone tissue down to the capillary and cellular level respectively in a single measurement.

MATERIALS AND METHODS

Animals

Hind limbs of adult C57BL/6J mice (N = 60, in house breeding) were employed for the development of the two VCC protocols. Additionally, C57BL/6J mice (N = 5) (RCC Ltd, Fuellinsdorf, Switzerland) were dedicated to the examination of the VCP protocol.

Materials

For the two VCC protocols, a polyurethane-based casting resin (PU4ii, vasQtec, Zurich, Switzerland) was used, which shows low viscosity, timely polymerization, and minimal shrinking and thus, provides high quality casts, including the finest capillaries (Krucker et al., 2006; Meyer et al., 2007). For the VCP protocol, barium sulfate (BaSO4) particles (Sachtleben Chemie GmbH, Duisburg, Germany) with a mean particle size less than 1 μm were dispersed for 10 min using a conical bead mill (Bühler AG, Uzwil, Switzerland) in suspension. Before injection, 3% bovine gelatin was added. The BaSO4 concentration of the injected suspension was 500 mg/mL. For concentrations higher than 500 mg/mL, the decreased BaSO4 particle size led to a significant increase in viscosity, up to a gel-like fluid, which made perfusion impossible. Therefore, the particle size has been carefully optimized to reach a viscosity similar to that of blood. To test for homogeneous contrast distribution, the intravascular BaSO4 perfusion was assessed using light microscopy for a subsequent D'Agostino-Pearson normality test (MATLAB R2006b; The MathWorks, Inc., Natick, MA).

Animal Preparation

For the VCC protocols, the animal preparation was described in detail elsewhere (Krucker et al., 2004). Briefly, the mice were deeply anesthetized with 100 mg/kg pentobarbital and intracardially perfused through the left ventricle with heparinized artificial cerebro-spinal fluid (ACSF). ACSF was perfused instead of the more common phosphate buffered saline (PBS) since for these animals, brain vascular corrosion casts were used for another study (Krucker et al., 2006). The perfusion (20–30 mL) was continued for 2–5 min at a constant rate (5 mL/min) and immediately followed by injecting 4% paraformaldehyde (PFA) in PBS (20–30 mL) at the same rate for prefixation of the vascular system. Subsequently, the PU4ii resin (<10 mL) was infused at a lower rate (2.7 mL/min). For the VCP protocol, deeply anesthetized mice were transcardially perfused with heparinized PBS followed by 4% PFA (same volume and rate as in the VCC protocol). Then, the dispersed BaSO4 suspension was injected (~5 mL in 1–2 min). No differences in vascular replica quality were observed due to the differences in the initial perfusion media. The bodies were kept at 5°C for a few hours before one limb of each mouse was dissected, skinned, and stored in 4% PFA. All animal preparation was performed by trained and licensed personnel and was in accordance with federal and institutional guidelines.

Vascular Replica Protocols

The two VCC protocols begin similarly with corrosion (maceration) of the soft tissue, before they branch off into two distinct protocols. The first VCC protocol (I) represents an adaptation of the classical VCC approach, where the soft and bone tissues were
resorbed and the remaining vascular replica was stained and freeze-dried for CT imaging. The second more advanced VCC protocol (II) involved bone decalcification, delipidation of the marrow cavity, and freeze-drying of the sample. In the following, the two VCC protocols are presented in detail. The VCP protocol required no further specimen treatment in addition to the animal preparation steps described before.

Maceration. The absorption of the casting medium (PU4ii) and the soft tissue of the murine hind limbs were very similar for X-rays at the energy spectrum covered by the μCT systems (around 30 keV). Therefore, for both VCC protocols, soft tissue was corroded (macerated) for X-ray absorption-based μCT assessment and segmentation of the vascular corrosion cast by digital thresholding of the 3D tomographic dataset. Two different solutions for maceration of skinned hind limbs were tested, 2% Alcalase® (Novozymes A/S, Bagsvaerd, Denmark) and 7.5% potassium hydroxide (KOH) (Fluka Chemie GmbH, Buchs, Switzerland): immersing in Alcalase® and KOH did not result in any changes in PU quality over time as verified by visual inspection using a stereo microscope.

VCC Protocol I. For investigation of the vascular corrosion cast only, bone needed to be separated before μCT scanning (bone resorption). For this purpose, specimens were immersed in 5% formic acid (Fluka Chemie GmbH, Buchs, Switzerland). Immersing in formic acid changed neither the morphology nor the quality of the PU over time, as verified by visual inspection under a stereo microscope. In order to increase the signal-to-noise ratio (SNR) and to reduce CT scanning time, the PU corrosion casts were immersed in 2% osmium tetroxide (OsO₄) solution (Riew and Smith, 1971) and were mounted on a rocking shaker for 48 h (staining). The staining solution was prepared earlier by dissolving osmium crystals (Electron Microscopy Sciences, Hatfield, PA, USA) in water during several days at 4 °C. OsO₄ is a widely used staining agent applied in transmission electron microscopy (TEM) to provide contrast to the image. Prior to freeze-drying (lyophilization), macerated specimens were rinsed gently and frozen in water. The specimens were then lyophilized (Christ, Alpha 1-2LD; Kühner AG, Birsfelden, Switzerland) for one day.

VCC Protocol II. Macerated specimens were decalcified using 14% ethylenediamine tetra-acetic acid (EDTA) at pH = 7.3 for 1 day (Kiviranta et al., 1980). Because the goal was to map the complete bone vascularization, the vascular corrosion cast needed to be assessed in the shaft of long bones (medullary cavity), where the vasculature is imbedded in the bone marrow. To provide optimal conditions for vascular corrosion cast segmentation within the medullary cavity, delipidation was necessary. In this process, marrow fat is removed and the differential between the reconstructed vascular corrosion cast and its surroundings in the reconstructed tomographic data set is increased. Therefore, chemical bone delipidation using different organic solvents (Beaupied et al., 2006; Chappard et al., 2004, 2006; Johnson et al., 2000; Moreau et al., 2000; Pruss et al., 1999; Worth et al., 2005) was tested. To test for possible physical damage, blue PU was submerged for several days in acetone, butane, chloroform, dichloromethane, ethanol, methanol, and methyl salicylate before measuring with μCT. Second, bone delipidation was employed after decalcification and prior to lyophilization to visualize properly the bone tissue and the vasculature within the medullary cavity at the same time. Finally, the specimens were rinsed gently in water and air-dried to extract residual solvents, before they were rehydrated for later lyophilization as described in the VCC protocol I.

CT Imaging and SEM

Specimens of the VCC protocol I were measured using a μCT40 desktop scanner (Scanco Medical AG, Brüttisellen, Switzerland) at high resolution (10 μm voxel size, 1000 projections over a range of 180°) and at 50 kV peak voltage. Specimens were imaged a first time by taking the average gray values of 10 projections at every angular position (FA). The second time after staining and without frame averaging (FA). With this setup, one measurement with and without FA took eight hours or less than one hour, respectively. Reconstructions were conducted using filtered backprojection. After segmentation by means of global thresholding, component labeling eliminated small artifacts or fragments separated from the sample.

To verify that the corrosion cast penetrated the cortical bone and reached the medullary cavity in the case of the VCC protocol II, where the murine bone and its vasculature was assessed concurrently, six specimens were prepared for SEM (Hitachi S-4000; Hitachi High-Technologies, Krefeld, Germany) by gold sputtering. In addition, the cortical shell of three bones was broken intentionally before sputtering to provide direct access to the medullary cavity using SEM. The other specimens of the VCC II and the VCP protocol were assessed by SR-based μCT. In comparison to conventional μCT, SR μCT provides higher SNR at increased resolution, which was important in this study for the examination of the devised VCC II and VCP protocol. SR μCT measurements were performed in air at the X-ray Tomographic Microscopy (XTM) station of the Materials Science (MS) beamline and at the beamline for tomographic microscopy and coherent radiology experiments (TOMCAT) at the Swiss Light Source (SLS) (Patterson et al., 2005; Stamparoni et al., 2002, 2007). The coherence of the synchrotron light was reduced by design using a rotating paper filter to flatten the illumination of the field of view (FOV). For both setups, 1001 projections were acquired over a range of 180° at a photon energy of 17.5 keV. The data was reconstructed using filtered backprojection. Measurements were performed in two different modes, in a global and local CT setup with corresponding nominal resolutions of 3.7 and 1.4 μm, respectively. In the conventional (i.e., global) CT setup, the projections of the entire specimen were recorded. This is in contrast to the local CT setup, where the specimen was bigger than the recorded FOV perpendicular to the rotation axis and therefore, only a portion of the whole sample was assessed. This local setup was required for high-resolution μCT (nominal resolution of 1.4 μm) without destruction of the specimen.
To partially suppress noise within the reconstructed data sets in both the global and local CT setup a constrained Gaussian filter (σ = 1.2; filter support = 1.0) was applied (Müller et al., 1994). After segmentation of the bone tissue and the vascular corrosion cast by means of global thresholding, component labeling eliminated small artifacts or fragments separated from the sample. The canal network and the osteocyte lacunae were segmented by means of negative imaging. In this context, negative imaging denotes the technique to first measure the bone matrix using CT, and subsequently, to extract the enclosed porosity as a negative imprint of the surrounding matrix. The intracortical porosity was then subdivided into the canal network and the osteocyte lacunar system (Schneider et al., 2007). For quantitative comparison between the canal network and the vascular replica of the VCP protocol assessed by global SR μCT, we quantified them by standard and element-based morphometry as described elsewhere (Schneider et al., 2007). In particular, we calculated canal volume density (Ca.V/Ct.TV), mean canal volume ((Ca.V)), mean canal diameter ((Ca.Dm)), and mean canal length ((Ca.Le)) for the canal network and the corresponding morphometric indices V.V/Ct.TV, (V.V), V.V/Ct.TV, (V.V), V.V/Ct.TV, (V.V), and (V.Le) for the vascular replica, where element-based indices (marked with brackets ( )) are given as mean values over the total number of elements. The vascular replica of the VCP protocol were evaluated first exclusively in the cortical bone (region 1) for direct comparison with the canal network and secondly, as a whole in the medullary cavity and the cortical bone together (region 2). Since by design, the intracortical canal network is formed by the cortical bone (region 1), it was only evaluated there. Finally, differences between the vascular indices in regions 1 and 2 and between the cannular and vascular morphometric measures in region 1 were compared using unpaired t-tests.

RESULTS
Vascular Replica Protocols
Maceration. A time series (N = 10) demonstrated that seven to eight hours were sufficient for complete maceration of the soft tissue of mouse hind limbs (Fig. 1A) using KOH, whereas full maceration was not achieved before 36 h in the case where Alcalase® was employed as maceration solution. Figure 1B shows mouse hind limbs after successful maceration. No difference in X-ray absorption was observed compared to untreated bone; neither for specimens corroded using KOH, nor for samples treated with Alcalase® (data not shown here).

VCC Protocol I. Depending on their size, bones of murine hind limbs were resorbed completely after not more than 18 h when using 5% formic acid. Figure 1C represents mouse hind limbs after successful soft tissue corrosion and bone resorption. In Figure 1D, the corresponding reconstructed tomographic data of the stained sample is given. The specimen was measured a first time before staining without and with 10 times FA. Component labeling of the 3D data sets revealed that number of disjoint vascular components was reduced by more than a factor of 10 and that the biggest vascular component accounted for more than 90% of all voxels when the scan was operated in the FA mode. In a second step, the same sample was stained and measured again without FA. Component labeling in the same region of interest (Fig. 1D) showed that the number of disjoint vascular components was reduced by more than a factor of 20 compared to the unstained specimen.

VCC Protocol II. Figures 2A–2C display one specimen in the region above the knee that has been imaged using SEM at different magnification levels. The corrosion cast penetrated the cortical bone, as it was observed for all six specimens analyzed by SEM. Furthermore, the corrosion cast was shown to reach the medullary cavity for all bones whose cortical shell was broken intentionally to provide direct insight. An example is given in Figures 2D–2F, where the casted vasculature within the broken neck of femur can be studied. Relating to the actual VCC protocol II, over-decalcification was not an issue for decalcification with EDTA. We also tested a commercial decalifier (Decal®; quartett Immunodiagnostika Biotechnologie Produktion und Vertriebs GmbH, Berlin, Germany), which is more aggressive than EDTA. A time series analysis (N = 10) performed at the murine femoral mid-diaphysis using μCT indicated that the cortical bone was entirely decalcified using Decal® within 15 min and that subsequently, over-decalcification damaged the bone tissue substantially by partial resorption. For that reason, mild decalcifiers should be employed to prevent potential over-decalcification. In Figure 3, one specimen is shown after corrosion, decalcification, and freeze-drying; particularly in the region of the femoral mid-diaphysis (Fig. 3A) and the femoral head (Fig. 3B). Already at low resolution (10 μm voxel size) using μCT (Figs. 3A and 3B) it became clear that marrow fat within the medullary cavity impeded proper vessel segmentation due to comparable X-ray absorption of the marrow fat and the casting material (PU4ii). During delipidation, PU became slightly less elastic and occasionally very brittle when immersed in acetone or chloroform, respectively. Moreover, it began dissolving and therefore lost resistance to a certain extent in ethanol, and more pronounced in methanol. For butane, dichloromethane, and methyl salicylate, the PU became brittle—in particular for dichloromethane—and shrank at the same time. In view of the fact that PU4ii is a polyurethane elastomer (Meyer et al., 2007) and given that solvents can attack plastic, their detrimental influence on PU4ii becomes clear. Ultrasonication, which can help removing fat traces mechanically, was not employed as it can introduce bone damage (Johnson et al., 2000). Finally, care needed to be taken when freeze-drying the macerated specimens as bone tissue became brittle when treated with KOH and, splintered during freeze-drying (Fig. 4). In Figure 5, one specimen is displayed after preparation according to the VCC protocol II as assessed with the highest achievable nominal resolution for global SR μCT in this study (3.7 μm). The marrow fat was effectively extracted and did not interfere with proper segmentation of the vascular corrosion cast, as it was previously observed for nondelipidated specimens (Figs. 3A and 3B).
**VCP Protocol.** The intravascular distribution of the BaSO₄ particles is shown in Figure 6, which was assessed using light microscopy. A D'Agostino-Pearson normality test indicated that the BaSO₄ particles were normally distributed ($P < 0.05$), which led to a homogenous contrast distribution, as required for quantitative assessment of the vasculature using X-ray-based CT. One specimen filled with the BaSO₄ suspension is depicted in Figure 7A, which was measured by means of local SR μCT. The vascular corrosion cast within the marrow cavity and the cortical bone could be successfully assessed. For the purpose of comparison, the canal network within the cortical bone (Fig. 7B) was derived by negative imaging.

**Morphometry**

The quantitative comparison of the morphometric measures for the canal network and the vascular replica is given in Tables 1 and 2. All element-based indices of the vascular replica (($V.V$), ($V.Dm$), and ($V.Le$)) were larger in region 2 (cortical bone + medullary cavity) in comparison with region 1 (cortical bone), which turned out to be statistically significant for ($V.V$) ($P < 0.002$) and with a trend for ($V.Dm$) ($P = 0.06$). In addition, the vascular volume density ($V.V/\text{Ct.TV}$) was found to add up to 10% of the canal volume density ($\text{Ca.V}/\text{Ct.TV}$) (cf. Fig. 7) within the cortical bone (region 1). In the same region, no significant differences between the canal and the vascular indices were found ($P > 0.05$). However, all element-based morphometric indices, describing the mean extension of single cannular and vascular elements, were systematically larger for the vascular replica compared to the canal network.

**DISCUSSION**

Because there is growing evidence for a close functional relationship between osteogenesis and angiogenesis as well as between bone remodeling and bone vascularization, there is a need for visual inspection and

---

**Fig. 1.** Mouse hind limbs before (A) and after (B) soft tissue corrosion (maceration) in 7.5% KOH at 58°C. A, B: The intact vascular corrosion cast (PU4ii) and the bone tissue are clearly visible. Mouse hind limbs after soft tissue corrosion (maceration) and bone resorption using 5% formic acid (C), where the display detail shows the same specimen after staining with 2% osmium tetroxide in the region of the femur (VCC protocol I). D: Reconstructed μCT data of stained vascular corrosion cast. C, D: Data were assessed by μCT at 10 μm nominal resolution.
Fig. 2. A–C: Murine specimen in the region above the knee after soft tissue corrosion, decalcification, delipidation, and freeze-drying (VCC protocol II). The corrosion cast penetrated the cortical bone as expected. Data were assessed by SEM at (A) ×22, (B) ×90, and (C) ×330 magnification. D–F: Murine specimen in the region of the neck of femur after soft tissue corrosion, decalcification, delipidation, and freeze-drying (VCC protocol II). The cortical shell of the bone was broken intentionally to provide direct insight into the medullary cavity, which proved that the corrosion cast reached also the inside of the bone. Data were assessed by SEM at (D) ×19, (E) ×65, and (F) ×120 magnification.
quantitative analysis of bone vasculature. We therefore implemented three different protocols for vascular replicas and devised CT methods for a true 3D, direct, and simultaneous measurement of vascular morphology and bone tissue down to the capillary and cellular level. Moreover, vascular morphology was examined in the light of the results based on a previously established method, where the canal network of cortical bone was derived only indirectly from cortical porosity.

Vascular Replica Protocols

VCC Protocol I. Soft tissue corrosion of the specimens should be performed using mild agents such as Alcalase® at moderate alkalinity if the bone tissue has to be preserved. Otherwise, there is the risk that the bone tissue will be irreversibly damaged during lyophilization. Further, staining with OsO4 largely increases the SNR in the reconstructed tomograms. In contrast, measurements with comparable SNR of unstained specimens would result in very high FA numbers and with that, in long scanning times on the order of several days. For that reason, treatment of the vascular corrosion cast with a highly X-ray absorbing contrast agent such as OsO4 can help reducing scanning time on the one hand, and increasing SNR on the other, also enabling proper segmentation of the vascular corrosion cast (Figs. 1C and 1D), which is a prerequisite for morphometrical quantification. Unfortunately, OsO4 is toxic as well as expensive and thus, should be replaced in the future by a harmless and cheaper staining agent if possible. Moreover, it became difficult to orient the vascular corrosion cast after bone resorption, and almost impossible to decide whether a vessel ran within the medullary cavity, within cortical bone, or actually was outside of the bone tissue.

CT measurements of macerated specimens revealed that simultaneous assessment of the bone tissue and the vascular corrosion cast was not feasible without any further specimen treatment as the highly absorbing bone tis-
sue masked the low absorbing vascular corrosion cast during tomographic reconstruction. This effect was probably due to the beam hardening correction, which is included in the filtered backprojection algorithms used by conventional \( \mu \text{CT} \) desktop scanners and which can only be adjusted for one tissue or material at the same time. Decalcification before lyophilization can solve this problem (VCC protocol II) by dissolving calcium stored in the bones and thereby, lowering their X-ray absorption.

VCC Protocol II. The X-ray absorption of bone can successfully be lowered, using preferably a mild decalciﬁer such as 14% EDTA. Lowering the X-ray absorption of the bone tissue by means of decalcification represents an effective method for the simultaneous assessment of the vascular corrosion cast and the bone tissue using CT (Figs. 3A and 3B). Finally, soft solvents such as acetone, which impaired neither the composition and morphology of bone, nor the integrity of the casting material, can help delipidating specimens (Fig. 5), particularly in the medullary cavity of long bones, where marrow fat can interfere with proper segmentation of the vascular corrosion cast.

VCP Protocol. Although the vascular corrosion cast and the bone tissue were available at the same time for the VCC protocol II, they could not be separated through segmentation based on global thresholding, given that the lowered X-ray absorption of the bone tissue approaches those values for the vascular corrosion cast material (PU4ii). In Figure 5, the interweavement of vascular corrosion cast, cortical, and trabecular bone can largely be discerned by an operator familiar with the murine bone microarchitecture and ultrastructure. Yet, many of these components are continuously connected with each other and thus, cannot be separated automatically by component labeling. Therefore, human operator interaction for segmentation by hand is necessary, which is time-consuming and beyond, leads to nonreproducible morphometric results. An alternative approach - which may solve this problem - is to inject a suitable contrast agent to the vascular system in vivo (Kiessling et al., 2004) as it was done in this study by using a customized \( \text{BaSO}_4 \) suspension. In this manner, the vascular replica and the bone tissue could be isolated by simple global thresholding (Fig. 7A). In addition, it must be pointed out here that compared to the VCC protocols, no additional sample preparation step was required after dissection of the murine limb. In such a way, not only time is saved, but also experimental uncertainties with respect to the morphology and integrity of the vascular corrosion cast can be avoided, which are usually involved in every VCC protocol.

Morphometry

Compared to the assessment of the canal network by using negative imaging (Fig. 7B), all vascular replica protocols presented in this study (VCC I and II, and VCP) have the advantage that they provide direct methods for the 3D assessment of the vascular replica. All morphometric measures for the canal network reported in this study (Tables 1 and 2) correspond to previously reported values for the murine cannular morphology in the same region, i.e., the femoral mid-diaphysis (Schneider et al., 2007). Moreover, the smaller dimensions of the vascular elements in region 1 (cortical bone) compared to region 2 (cortical bone + medullary cavity) was due to the fact that the vasula-
ture is confined by the cortical bone in region 1, whereas large branches of the vasculature can be found within the marrow cavity and the cortical bone (semitransparent shell in white), which includes osteocyte lacunae (prolate ellipsoids in yellow). The vascular replica and the bone tissue were isolated by global thresholding.

Interestingly, all element-based morphometric indices within the cortical bone were found to be larger for the vascular replica in contrast to the canal network, yet not statistically significant. This is counterintuitive because the space between the cannular and the vascular surface is occupied by the endothelium, an ~1-μm-thick cell layer at the interior surface of the vascular system (van den Berg et al., 2006), and consequently, the extension of the cannular elements was expected to exceed the vascular dimensions. There are two possible explanations for this finding. First, only a fraction of the canal network was originally occupied by functional vessels in the animal, specifically by larger branches of the vasculature. The remaining volume of the canal network would then be empty and/or inhabited by bone remodeling units (including osteoclast lacunae). Secondly, the vascular contrast perfusion was not complete. For instance, round or tapered dead ends and the lack of endothelial imprints on the cast surface are well known indicators of incomplete intravascular filling.

**TABLE 1. Morphology of the canal network**

<table>
<thead>
<tr>
<th>Region</th>
<th>Ca.V/CT.TV (%)</th>
<th>(Ca.V) (10^{-3} mm³)</th>
<th>(Ca.Dm) (μm)</th>
<th>(Ca.Le) (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical bone</td>
<td>1.22 ± 0.26</td>
<td>19 ± 2</td>
<td>10.3 ± 0.4</td>
<td>89 ± 6</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation (N = 5).

Ca.V/CT.TV, canal volume density; (Ca.V), mean canal volume; (Ca.Dm), mean canal diameter; (Ca.Le), mean canal length.
(Lametschwandtner et al., 1990; Weber et al., 2008). Both assumptions are supported by the observation that the vascular volume density (V.V/Ct.TV) amounted to no more than 10% of the canal volume density (Ca.V/Ct.TV) (Tables 1 and 2). Eventually, as both explanations do not exclude each other, a combination of both factors may explain morphometric differences between the canal network and the vascular replica. Future investigations should unambiguously identify the actual factors that cause the observed difference between the extension of the cannular and vascular elements.

**Limitations**

There were several sources of error within the presented protocols, which can affect the morphometrical quantification of the bone tissue and the vascular replica. They are summarized in the following. First, scanning and reconstruction of the vascular corrosion cast according to VCC protocol I without prior staining leaves many disjoint vascular components. As a result, subsequent quantification provides wrong morphometrical measures of the vascular corrosion cast. On this account, staining with a highly X-ray absorbing contrast agent such as OsO₄ is a prerequisite for proper segmentation of the vascular corrosion cast for the VCC protocol I. Although the procedure is simple and straightforward, OsO₄ is unfortunately toxic and expensive and should be replaced at some point by a harmless and cheaper staining agent. Second, over-decalcification can happen in the VCC protocol II when using aggressive decalcifiers. Consequently, bone tissue integrity is impaired by partial resorption and the morphometric outcomes are distorted. For this reason, only mild decalcifiers should be used for decalcification, such as EDTA. Third, inappropriate selection of the solvent for delipidation in line with the VCC protocol II can particularly cause dissolving and shrinking of the polyurethane-based casting material, which introduces a bias of the derived vascular morphometric indices. Therefore, soft solvents must be used for delipidation, which do not alter the morphology of the vascular corrosion cast material. In this study, acetone turned out to be the optimal solvent since it did not change the morphology of the polyurethane-based casting material and only slightly reduced its elasticity. Finally, for both VCC protocols (I and II), human operator interaction was required for the orientation or segmentation of the vascular corrosion cast and the bone tissue. This leads to nonreproducible morphometric results. The VCP protocol overcomes this limitation, where the vascular replica and the bone tissue were separated automatically by simple global thresholding.

**CONCLUSIONS**

There is one published preclinical work (Duvall et al., 2004), where µCT at nominal resolutions between 16 and 36 μm along with VCC was used to analyze collateral vessel development after ischemic injury. In addition, barium sulfate as contrast agent for µCT at 70 μm nominal resolution has been used before in a preclinical study for in vivo imaging of tumor angiogenesis (Kiessling et al., 2004). In our basic research study, the goal was the development of several protocols for the concomitant quantification of the vasculature and the bone tissue at very high 1.4 and 3.7 μm nominal resolution, which allows for reliable 3D quantification and morphometric analysis of murine bone ultrastructure as recently introduced (Schneider et al., 2007).

In particular, we devised two different VCC protocols, identifying and handling a number of challenges during sample preparation. The first VCC protocol (I) represents an adaptation of the classical VCC approach, where the soft and bone tissues were resorbed and the remaining vascular replica was stained and freeze-dried for CT imaging. It is straightforward and thus, easy to implement. However, it was difficult to locate the vascular corrosion cast in relation to the resorbed bone, which makes it particularly problematical to compare quantitative measures of the vascular corrosion cast from different animals. The VCC protocol II solved this problem, where the bone tissue was decalcified for the concomitant µCT measurement of the bone tissue and the vascular corrosion cast. Unfortunately, the vascular corrosion cast and the decalcified bone tissue must then still be separated by a human operator since the two phases showed similar X-ray absorption and thus, comparable contrast in the reconstructed CT data. For this reason, we also adapted SR µCT methods to combine them with a novel contrast modality (VCP protocol), where a customized contrast suspension was directly injected to the vascular system in vivo. In this manner, vascular replicas were imaged and quantified successfully down to the capillary level with simultaneous measurement of the bone tissue down to a cellular regime. In addition, we could now separate the vascular replica and the bone tissue automatically by simple global thresholding due to the significant difference in X-ray absorption between the BaSO₄ suspension and the bone tissue. Another advantage of the VCP protocol was that compared to the VCC protocols, no additional sample preparation step was required after dissection of the murine bone.
rine limb. By this means, time was saved and several sources of error within the VCC protocols (see Limitations) were eliminated, which can affect the morphometrical quantification of the bone tissue and the vascular replica. One disadvantage of the VCP protocol is that the vascular replica is not readily available for other imaging modalities such as optical or electron microscopy. Since this is the case for the two VCC protocols, there is still a need to keep these protocols as well.

Eventually, cannular and vascular morphometric indices, as they were derived in this study, can now be related directly to measures of bone biomechanics, providing important insights in the structure function relationships between bone tissue and bone vascularization. Generally speaking, our presented protocols can be applied to study quantitatively the vascular pattern of many different normal and pathological organs and tissues in a truly 3D fashion (Lametschwandtner et al., 1990), such as in the brain, heart, kidney, lung, and the skin, to name only a few. In the long run, we believe that the presented framework of an extended VCC protocol and a novel VCP modality together with conventional as well as adapted SR-based CT methods can serve as a potent and complementary combination for simultaneous 3D quantitative morphometry of the bone tissue and its vascularization at the same time, providing new insights in the close relationship between bone tissue and the vascular network.

ACKNOWLEDGMENTS

We thank Dr. Johannes Vogel for the animal care, Dr. Bernhard Stalder from Bühler AG (Uzwil, Switzerland) for expert help in the BaSO₄ dispersion procedure, and Dr. Amelia Grosó for her support during the measurements at the Swiss Light Source.

REFERENCES


Microscopy Research and Technique