Sub-Lamellar Microcracking and Roles of Canaliculi in Human Cortical Bone

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Abstract

Bone is a tough biological material. It is generally accepted that bone's toughness arises from its unique hierarchical structure, which in turn facilitates distributed microcracking prior to fracture. Yet, there has been limited progress on the detailed roles of the structural elements in the microcracking process. The present study examines the structure - microcracking relations at the lamellar and sub-lamellar levels of human cortical bone subjected to compressive loading. Laser scanning confocal microscopy revealed a clear influence of the local structure and porosity of the Haversian systems' lamellae on microcrack development. In particular, crack initiation and growth under transverse compression were associated with stress concentration at canaliculi. Later stages of microcracking showed extensive sub-lamellar cracks forming cross-hatched patterns and regularly spaced 0.5 to 1.7 μm apart. The density, size and regularity of the crack patterns suggest enhanced inelastic deformation capacity through cracking control at the level of mineralized collagen fibril bundles. The present study thus improves the current understanding of the nature of inelastic deformation and microcracking in bone and further suggests that bone's resistance to fracture is achieved through microcrack control at multiple length scales.

Keywords: Bone fracture, Cortical bone, Inelastic deformation, Microcracking, Canaliculi, Confocal microscopy

Introduction

Bone is a nanocomposite of carbonated apatite nanocrystals and organic phases mainly composed of fibrous collagen protein. It is known for its unique hierarchical structure which has been hypothesized to give rise to its exceptional mechanical performance [1-4]. Although highly mineralized and with an extensive porosity network, human cortical bone exhibits remarkable inelastic deformation [4-8]. Such deformation is critical to its resistance to fracture as it relaxes stress concentrations [8-12]. However, how bone's different structural levels, from the mineralized collagen fibrils to the Haversian systems (or secondary osteons) and their lamellar organization, are involved in the deformation process is still poorly understood.

Bone's inelastic deformation at nano-scale has been associated with slip at mineral/collagen interfaces [7,13], increased energy dissipation arising from nanostructural heterogeneities [14], interfibrillar shear sliding [15-17], and unfolding of non-collagenous proteins (NCPs) [18,19]. At the micro-scale, the inelastic deformation is accompanied by distributed microcracks [6,20] which, in human cortical bone, are present within both interstitial and osteonal lamellae [21,22]. Microcrack initiation has been linked to Haversian canals [21,23] and osteocyte lacunae [24,25]. Long microcracks (~100 μm), forming at later stages of cracking [26], interact with osteons [27] and the hyper-mineralized [28] cement lines [29,30]. "Bridges" at various length scales also hinder crack growth [11,31,32]. Despite these progresses, the nature of microcracking (distributed microcracks) at the lamellar, sub-lamellar, and fibrillar levels is still largely unknown [8,33,34].

Our recent study has shown the role of the osteonal lamellae in redistributing stress around each Haversian canal through the stable development of multiple intralamellar microcracks [8]. However, where those cracks initiated and how they developed within the bulk remained unclear [8,34]. Canaliculi are fine channels (~200 nm) [35] connecting the osteocyte lacunae together. Despite their high distribution density (1×10^6 canaliculi mm^{-3} [1]) and potential as stress concentrators [36], their roles in the microcracking process remain hypothetical [37,38]. A detailed study of bone's microcracking process would clarify the relations between microcracks and those fine structures.

The present microscopy study focused on the structure - microcracking relations at the lamellar and sub-lamellar levels of human cortical bone. The purpose was to investigate the morphology and the development of microcracks within the osteonal lamellar structure and with respect to the canaliculi network. This was carried out through imaging with a laser scanning confocal microscope following longitudinal and transverse compressive loading. The work is the first step to bridge the gap between microcracking and nano-scale deformation mechanisms. It provides insight into the design strategies used in bone to resist fracture.

Materials and Methods

The present study investigated the microcracking process under compression in seventeen human cortical bone specimens (ten transversely and seven longitudinally compressed; Fig. 1). Mechanical characteristics and initial fluorescence microscopy analyses have been reported previously [8,34]. Figure 1 summarizes the experimental procedures described in details in the following sections. All studies were approved by the Clinical Research Ethics Review Board at the University of British Columbia.

Specimens and Mechanical Testing

As formerly described [8,34], the seventeen cortical bone specimens were extracted from three unembalmed human cadaveric femoral shafts taken from healthy donors (2 males, 64 and 69 years old; 1 female, 55 years old), free from metabolic bone diseases. They were mechanically ground (Beta, Buehler) into rectangular prisms, polished on all surfaces using diamond suspensions down to 1.0 μm , and vibration polished (Vibromet 2, Buehler) using 0.05 μm colloidal silica suspension down to their final dimensions of either 3 mm $\times$ 3 mm $\times$ 6 mm or 2.5 mm $\times$ 2.5 mm $\times$ 5 mm. The specimens were then loaded under monotonic (fourteen specimens) or step-wise (three specimens) compression (Instron 8874, 25 kN load cell or Minimat Materials Tester 2000, 1 kN load cell) at a crosshead speed of 0.1 mm/min under wet conditions (Fig. 1). For each orientation, two specimens were loaded to fracture and five specimens were unloaded before fracture (i.e., at about 5% drop from peak load) to better analyze microcracking. The

extent of damage was assessed by counting the proportions of damaged osteons from the middle third of the latter specimens.

Laser Scanning Confocal Imaging Analysis

Following mechanical testing, all specimens were stained with fluorescein (Fisher Scientific) in order to label the microcracks (Fig. 1). The dye also stained the specimens' original surfaces and porosity network. Sixteen (nine transverse and seven longitudinal) specimens were dehydrated and stained according to a procedure described elsewhere [21]. Briefly, the specimens were sequentially immersed into acetone and a series of ethanol/water solutions (80%, 90%, and 100%) for periods of 24 hours per step, followed by overnight staining under vacuum in a filtered saturated solution of fluorescein and 70% ethanol. They were then taken through various preparation and imaging steps to characterize the microcracking. The remaining specimen was stained for 30 minutes in an aqueous solution of 2% fluorescein, rinsed 10 minutes in tap water, and directly taken to LSCM for examination under water immersion (both specimen and objective lens) in order to verify if secondary staining occurred during subsequent preparation steps.

Following staining, all specimens were examined under the optical microscope using reflected white light (BF; Nikon Eclipse LV100) as well as epi-fluorescence light (EF; Nikon Eclipse E600) with excitation at approximately 490 nm and emission at approximately 525 nm (Fig. 1). Four transversely compressed specimens were ground and polished to their central sections (two were ground normally to the osteons and two parallel to the osteons) to characterize microcracking within the bulk. Specific sites were imaged at higher magnification under the laser scanning confocal microscope (LSCM; Olympus FluoView FV1000, Olympus Canada Inc., Markham, Canada). Three specimens were sputter-coated with a thin layer of gold for scanning electron microscope (SEM; Hitachi S-3000N and/or Hitachi S-4700, Hitachi Ltd., Tokyo, Japan) examination. These steps (Fig. 1) allowed for the description of microcracks' morphology and development in relations to bone's hierarchical structures (osteons, osteonal lamellae, osteocyte lacunae, canaliculi, etc.).

LSCM imaging was carried out under oil immersion (objective lens) while the specimens, resting on the cover slide of an in-house built chamber, were immersed in pure ethanol (Commercial Alcohols Inc.) with the exception of one specimen which was immersed in water (see above; [Fig. 1](#)). The 488 nm line of a multi-line Argon laser was used for excitation and the specimens emitted at 519 nm. The z-step (z-axis defined as the image depth) was set at 200 nm (resolution in z of approximately 587 nm) and the specimens were typically imaged to a depth of 20 μm starting at the surface. A 20 μm z-stack thus consisted of a series of 100 images, each 200 nm apart (but capturing light from 587 nm thick planes), to a depth of 20 μm . Hence, three-dimensional (3D) information of the cracking and the porosity network was obtained. All measurements were calibrated based on 200 nm diameter fluorescent microspheres images (TetraSpeck microspheres 0.2 μm , Molecular Probes Inc.).

All z-stack images were reviewed qualitatively for microcracks' shape and approximate dimensions. As the canaliculi were suspected to influence transverse compression microcracking, cracks and canaliculi distributions were analyzed using ImageJ 1.43s (National Institutes of Health; USA). Sub-lamellar crack spacing was measured in fourteen damaged regions from seven different specimens (two regions or images per specimens). Similarly, fourteen other regions were used to characterize the canaliculi distribution. Canaliculi density was obtained by counting the intensity peaks of profiles plotted along three lines on 15 μm z-stack images (accumulation of virtual slices displayed as one slice; e.g., [Fig. 4a](#)) and dividing the count by the volume subtended by each line. Canaliculi spacing was obtained by digitally measuring the distance between the intensity peaks along the same lines, but for a single thin plane of approximately 587 nm (approximate resolution in z). Canaliculi volume fraction (vol%) was estimated based on their averaged measured density and reported diameter [35]. Cracks and canaliculi spacings were compared using a t-test (Primer of Biostatistics version 6.0; McGraw-Hill Companies, USA) with a confidence level of 95% ($p < 0.05$).

Results

Multi-Scale Cracking in Bone: The Advantages of LSCM

Transverse Compression Cracking

Transverse compression loading resulted in a fracture plane oblique to the loading direction and along the length of the osteons (Figs. 2a-b). At the osteonal-interstitial level, extensive cross-hatched damage (80 ± 5 % of osteons were damaged) was homogeneously distributed within the bulk (Fig. 2c). At the level of a single osteon (Figs. 2d-e), each set of cross-hatched cracks consisted of groups of intralamellar (within the bright/thick layers where fibrils are predominantly longitudinally aligned [39-41]) arc-shaped microcracks radiating out into the quadrants along oblique directions from the Haversian canal, as reported previously [8,34]. Under the fluorescence microscope (EF) where background light compromises resolution, arc-shaped microcracking appeared as single, blurry cracks inside each lamella (Fig. 2f). Very interestingly, the damage inside each arc-shaped microcrack was resolved into finer cracks under the LSCM (Fig. 2g). These intralamellar microcracks also formed a cross-hatched pattern (Fig. 2g) composed of relatively long circumferentially oriented cracks, 0.9 ± 0.2 μm (range: 0.7 to 1.7 μm) apart, and radially oriented cracks, 0.8 ± 0.1 μm (range: 0.5 to 1.2 μm) apart, with lengths ranging from below 1 μm up to about 10 μm .

The detailed morphology was generally similar from one osteon to another. The intralamellar microcracking varied in the extent of circumferential and radial cracking and the orientation of radial cracks. Dark layer damage (e.g., Fig. 4e) and flame-like cracking at the ends of circumferential microcracks were also often observed (Supplementary Material Fig. S1). Those variations occurred due to either the stage of damage development or slight changes in the local stress field (e.g., location within the shear band, events at the crack tip, near-surface effects, etc.). Water immersion LSCM imaging of the fresh (non-dehydrated and stained in an aqueous fluorescein solution) specimen showed the same features as dehydrated specimens subjected to post-staining preparation steps (Fig. 1). All microcracking morphologies of transversely compressed bone (presented in Figs. 2, 4, and S1) were thus non-artifactual.

Longitudinal Compression Cracking

The fracture following longitudinal compression was oblique to the osteons (Figs. 3a-b). At the osteonal-interstitial level, the well-known cross-hatched microcracks were distributed in 69 ± 5 % of the osteons (Fig. 3c). Higher magnification observations revealed interactions with lamellar and sub-lamellar structures. Within the osteons, cross-hatched microdamage consisted of relatively long microcracks developing oblique to the lamellae (Fig. 3d). Although roughly linear, those cracks locally changed orientation between layers in a "stairway-like" manner (Fig. 3d), being more parallel to lamellar boundaries (and loading direction) within dark layers where fibrils are oriented along the osteons [40-42]. Uncracked ligaments/bundles (and lamellae), previously reported in the literature [11,31,32,37], were also mostly observed within these layers (Fig. 3d insert). High resolution LSCM images (Figs. 3e-g) further showed that the microcracks consisted of many smaller cracks, particularly near the stairway steps and the osteonal-interstitial boundary where the cracking morphology changed to arrays of small wavy and less oblique cracks spreading into a wider area ahead of the main crack tip. Further down in scale, longer microcracks near the Haversian canals would sometimes locally develop finer cracks (Fig. 3f), similar in size and spacing to those of transversely compressed bone. The microcracks also interacted with osteocyte lacunae (Fig. 3g), consistent with literature [24,25,43], and there were evidences of crack interactions with canaliculi which, in some cases, may have developed small cracks (Fig. 3g).

Bone lamellae influenced the microcrack paths and morphologies in specimens compressed along both orientations (Figs. 2e-g and 3d-e). However, at the resolution of LSCM, the structure - microcracking relations in longitudinally compressed specimens were not as easily recognizable (e.g., Fig. 3f) as in transversely compressed specimens. Therefore, as a first step to understand those relations, the latter group was further examined.

Bone's Porosity: The Canaliculi Network

Fluorescein also stained the densely distributed canaliculi network. As seen on a z-stack LSCM image of an intact osteon, the canaliculi distribution is uniform (Fig. 4a). The average canaliculi spacing was $4.3 \pm 1.4 \mu\text{m}$ and, in agreement with literature [1], the average osteonal canaliculi

density was $1.6 \pm 0.8 \times 10^6$ canaliculi/mm³. This would correspond to a volume fraction of about 5.0 %.

Sub-Lamellar Crack Initiation and Development

Although most osteons in transversely compressed specimens were damaged, they were at various stages of damage development. Even different quadrants of an osteon showed different stages (Fig. 2e). This allowed for the study of crack initiation and development. Figure 4a shows an intact osteon. Figures 4b to 4e present the cracking sequence within the bulk from early initiation to a late stage corresponding to shear band formation.

First, small circumferential cracks initiated at the canaliculi (Figs. 4b-c) within the bright layers of each osteonal quadrants. Interestingly, multiple crack nucleations not only occurred at numerous canaliculi but also along the length of a single canaliculus (Fig. 4b). Although also initiating cracks [8], osteocyte lacunae are far fewer (1.5×10^4 mm⁻³ [1]) than canaliculi and thus did not seem to play a critical role in the process. Following initiation, the cracks merged together forming longer circumferential cracks. As the main crack propagated, other cracks initiated at canaliculi ahead of the main crack tip and eventually merged with it (Fig. 4d). A similar merging mechanism also took place in the depth direction (Fig. 4d z-plane). Hence, the micro-scale arc-shaped cracks, formed from numerous finer cracks (Fig. 4e z-plane), penetrated deep into the lamellar structure taking a "sheet-like" (long but relatively flat) appearance that followed the curvature of the lamellar boundaries. Longitudinal sections' imaging also supports these observations indicating that crack growth also happened parallel to the osteons. As a single canaliculus could initiate many cracks, circumferential cracking usually occurred on more than one plane resulting in many parallel, but not necessarily independent (linked through the depth and/or via radial cracking), circumferential cracks. During the development of circumferential cracks, radial cracking also occurred and linked the circumferential cracks together (Figs. 2e-g and 4e). Those radial cracks differed from the radially oriented canaliculi. Their high distribution density could not be explained by the larger canaliculi spacing. This suggests a relation to the mineralized collagen fibrillar bundles. The next stage of damage development was less clear but could involve lengthening and widening of sub-lamellar cross-hatched (Figs. 2e and 4e). At

certain points, the damaged bright layers could not provide adequate support to the dark layer fibrils. This culminated into shear band formation at the osteonal level [8] through kink failure resulting in damage within the dark layers and a radially oriented microcrack at the Haversian canal (Fig. 4e).

Discussion

In the present study, LSCM following compressive loading enabled high resolution 3D imaging of sub-lamellar cracks within the bulk of human cortical bone (Figs. 2e-g and 3e-f-g). Transverse compression proved particularly valuable to examine the structure - microcracking relations at the lamellar and sub-lamellar levels. Multiple intralamellar cracks progressively developed within the osteonal wall (Figs. 2c-d-e). The initial stages involved multiple nucleations and crack coalescence through the canaliculi (Figs. 4b-c-d). Further, the process resulted in extensive sub-lamellar cross-hatched cracks regularly spaced 0.5 to 1.7 μm apart (Fig. 2g), a size comparable to mineralized collagen fibril bundles. The high crack density, also observed under longitudinal compression (Fig. 3f), suggests cracking control at the fibrillar level. Such fine damage could further stabilize microcrack growth at higher hierarchical levels. The findings hint at the nature of cracks in bone and the roles of canaliculi in bone's mechanical and remodeling responses.

Role of Canaliculi in Crack Initiation and Growth

Material failures are generally associated with high local stresses and strains around defects. In bone, high strains and microcrack initiation have been linked to Haversian canals [21,23] and osteocyte lacunae [24,25,44] while the roles of the highly populated canaliculi (Fig. 4a) have not been demonstrated. As illustrated in Figure 5a, the present study showed that in the bulk of transversely compressed bone, multiple short circumferential cracks initiated at the canaliculi (Figs. 4b-c). Those cracks were located within the high interlamellar shear zones (Fig. 5a; shear normal and parallel to the lamellae, i.e., along and across the canaliculi) surrounding the Haversian canals [8]. They eventually merged together (Fig. 4d), by a process similar to shear micro-voids coalescence in metals [45], and developed into long, roughly parallel cracks along both the circumferential and longitudinal (depth) osteonal directions (Fig. 5a; y and z directions,

respectively). A 3D mechanical simulation (ABAQUS/CAE Version 6.7-5; Dassault Systèmes) of a canaliculus under pure shear revealed 1.4 times increases in local elastic stresses and strains along the canaliculi z-axis (depth direction parallel to osteons) and spanning their entire length. Weak sites at these locations would likely initiate cracking. This corresponds well with the crack initiation observations (Fig. 4c) and supports the merging process along the depth direction (Fig. 4d). Note that the process described here within the bulk is different from near-surface events where radial cracks develop into circumferential microcracks [8]. Although based on many repeated observations, the sequence presented also assumed that the intralamellar microcracking morphological variations represented different stages of the same process. Such an assumption was necessary in view of the technical difficulties (e.g., events happening within the bulk, necessity to label microcracks, resolution of LSCM with water immersion, photobleaching, etc.) associated with monitoring the progress of individual microcracks.

Longitudinal compression seemed less sensitive to canaliculi (rather being more sensitive to osteocyte lacunae (Fig. 3g) [25] and Haversian canals [21]) as few possible cases of canaliculi crack initiation were found (Fig. 3g). Nonetheless, considering their orientation [36], the canaliculi may have similar roles in other loading modes. They have been suspected to be involved in fracture under longitudinal tension [37] and to interact with diffuse damage in human bone [38]. Torsion loading, for which inelastic strains have been associated with circular cracking [46], introduces a similar interlamellar shear state within the osteonal wall (Fig. 5a) and hence is likely to involve canaliculi. Pre-existing microcracks, generally attributed to in vivo fatigue [47,48], are thought to contribute to fragility and stress fractures [49,50]. Those were examined in our previous study [8]. Upon re-visiting the BF and EF images, more than half were found to be parallel to interstitial lamellae boundaries, consistent with others' observations [29,48]. Although difficult to verify, this suggests that canaliculi may be similarly involved in initiation and growth of microcracks in vivo.

Possible Role of Canaliculi in the Remodeling Process

One characteristic that distinguish human cortical bone from other hard tissues such as dentin is its ability to adapt to mechanical loads through remodeling [1,4]. High strains [44,51] and

microcracks [25,47,52-55] have been proposed as stimuli for mechanosensation through disturbances of lacuno-canalicular network fluid flow [56-59] and/or osteocyte syncytium integrity [55,58,60]. Long microcracks have been shown to rupture the osteocyte processes within the canaliculi network [61]. Interestingly, a recent study showed that there is a decrease in osteocyte lacunar density in aged human bone and proposed that this may cause deteriorations in the canalicular fluid flow, reduce the detection of microdamage, and lead to failure and delay of remodeling [62]. Our results support these theories. We hypothesize that canaliculi are directly involved in the remodeling process through initiating microcracking. As cracking at the canaliculi would disturb the lacuno-canalicular fluid flow or rupture the osteocyte processes, the canaliculi, with their uniform distribution and high density (Fig. 4a) [1], would indeed be well-organized for damage detection. According to the canaliculi spacing results, any crack longer than about 6 μm (consider the size of a single crack in tensile "diffuse" damage) would encounter a canaliculus. This may thus represent an early damage detection strategy used by osteocytes to activate the remodeling response. In the aging population where the osteocyte lacunar density is low [62], the canalicular density would be decreased accordingly. This could lead to deficient damage detection, excessive microcrack accumulation, and increased bone fragility.

Sub-Lamellar Cracking: Towards the Nature of Cracks in Bone

All cracks start at bone's lower hierarchical levels. Recent studies have shown the involvement of mineral/collagen interfaces [7,13], NCPs [18,19,32], nano-scale heterogeneity [14], and mineralized collagen fibrils [15-17] in bone's deformation process. It is challenging to relate microcracks to such fine structures. LSCM has proven a powerful technique to investigate sub-microscopic bone cracks and porosity [20,33,38,63]. Its resolution approaches the size of the mineralized collagen fibrils. For both loading orientations, the present LSCM study revealed a clear sub-lamellar cross-hatched pattern involving high density of very fine cracks (Figs. 2g and 3f).

Transverse compressive loading showed a particularly clear relation with the local structure of each lamella. Extensive cracking developed intralamellarly within the osteonal bright layers (Figs. 2d-e-g). Considering the local fibrillar orientation parallel to the osteons, the regular crack

spacing, which was smaller than the canaliculi spacing, suggests that a repeating structure of about 800 nm in size, such as the mineralized collagen fibril bundles, would govern the cracking. The radial cracks are reminiscent of matrix cracks in 2D fiber-reinforced composites under interlaminar shear [9]. This similarity explains their intralamellar confinement. The cracks evolved without significant fibril interactions in the bright layer while fibrils impeded their growth (e.g., by bridging [31,32]) near the dark layer. The size and regularity of the sub-lamellar cross-hatched pattern further suggest an interfibrillar (as opposed to intrafibrillar) nature, akin to interfacial and matrix shear cracking in transversely compressed fiber-reinforced polymers [64]. This interpretation was proposed based on SEM observations of fractured trabecular bone [32]. It is also well-supported by TEM observations following single osteon loading [37,65]. Such scenario, illustrated in Figure 5b, would likely involve NCPs [18,19,32,66] and extrafibrillar minerals/proteins interfaces [12,67]. Further work is planned to directly link sub-lamellar cracks to the mineralized collagen fibrils and to consider other loading modes.

Mechanically, such high crack density could enhance the inelastic strain capacity of the lamellae (consider the strain in a solid containing many microcracks as compared to just a few [68]), further stabilizing the microcracking process at higher hierarchical levels. Interestingly, long interstitial microcracks, such as those pre-existing cracks commonly found in vivo [50,69,70], often extend to the osteons [27,29,49] and larger cracks usually demonstrate a process zone with damaged osteonal lamellae [21,38,71]. Intralamellar microcracking may be another mechanism (along with crack deflection [30] and bridging [31,32]) used in bone to hinder interstitial microcrack growth. Such strategy could provide both robustness to the material and stimulus for remodeling.

Conclusions

High resolution laser scanning confocal microscope observations of compressed human cortical bone enabled us to establish relations between microcracking and the osteons' lamellar and sub-lamellar structures. The numerous canaliculi initiated and facilitated the development of multiple intralamellar cracks. Such association implies stress concentration at canaliculi but may also be a strategy for early damage detection. The later stages of the microcracking process involved high

densities of sub-lamellar cracks forming regular cross-hatched patterns. Those patterns consisted of very fine cracks only a few microns in length and less than 1 μm apart. Their size and regularity could result from crack control at the level of the mineralized collagen fibrils. The present findings thus suggest that fracture resistance in bone is achieved by controlling microcrack development at multiple hierarchical levels. The study advances our knowledge on the nature of microcracks in bone and their link to the mineralized collagen fibrils.

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Figures

Fig. 1. Summary of experimental procedures. A total of 17 human cortical bone specimens were submitted to compressive loading along two different orientations. For each orientation, 2 specimens were loaded to fracture and 5 specimens were unloaded prior to fracture (i.e., at about 5% drop from peak load). The latter were also used to count the proportions of damaged osteons. For the transverse orientation, 3 additional specimens were loaded in a step-wise manner to monitor deformation and damage development under BF and EF (see [8] for details). All specimens were stained in an ethanol-based fluorescein solution except for one which was kept fresh (non-dehydrated, stained in a water-based solution of fluorescein, and imaged under water immersion). All specimens surfaces were examined under BF and EF and 4 specimens were subsequently ground and polished to their central section to examine microcracking within the bulk. LSCM imaging followed and images from 7 transversely compressed specimens were used to characterize the canaliculi and cracks distributions. Two regions (or images) per specimen were analyzed for a total of fourteen regions.

Fig. 2. Multi-scale microcracking in human cortical bone under transverse compression. (a) Schematics of the transverse (90°) loading orientation with respect to the osteons; (b) BF image of the macroscopic oblique fracture pattern; (c) EF image of distributed cross-hatched damage at the osteonal-interstitial level; (d) BF image of arc-shaped microcracks within the osteonal bright layers (arrowheads); (e) LSCM image showing multiple intralamellar cracks in the four quadrants of an osteon; (f-g) Low resolution EF and high resolution LSCM images taken from the lower left quadrant (dotted line) of the osteon in (e). LSCM reveals a sub-lamellar cross-hatched pattern composed of fine radially and circumferentially oriented cracks with spacing similar in size to fibrillar bundles. Compressive load applied vertically for a-b-c and horizontally for d-e-f-g.

Fig. 3. Multi-scale microcracking in human cortical bone under longitudinal compression. (a) Schematics of the longitudinal (0°) loading orientation with respect to the osteons; (b) Stereomicroscope image of the macroscopic oblique fracture pattern; (c) EF image of distributed cross-hatched damage at the osteonal-interstitial level. Note the morphological similarity with transverse compression damage; (d) Backscattered electron (BSE) micrograph of osteonal oblique microcrack formed at the Haversian canal (HC; approximated by dotted line) and extending to the boundary (approximated by dashed line) between osteonal (O) and interstitial (I) bone. Notice the "stairway-like" changes of orientations (arrowheads) with the layers as well as the uncracked ligaments (insert: empty arrowheads) and lamellae (arrows). Insert location shown by dotted line; (e) LSCM images showing smaller cracks with the larger oblique microcrack. The identified osteocyte lacunae (asterisks), ligament bridging (empty arrowheads) and full circle correspond to the same locations in (d). Note the change in crack morphology near the osteonal-interstitial boundary (bottom image); (f) High resolution 3D LSCM image of localized, finely spaced cross-hatched cracking near a Haversian canal (HC). The z-planes ("cut views" at locations shown by white lines) show deep oblique cracking; (g) LSCM images of crack interactions with osteocyte lacunae (asterisk) and canaliculi (double arrows). Compressive load applied vertically for a-b-c and horizontally for d-e-f-g.

Fig. 4. LSCM imaging of the canalicular network and the intralamellar cracking sequence within the bulk of transversely compressed bone specimens. (a) Image of a 5 μm deep z-stack showing the lacuno-canalicular network within an intact osteon; (b) Multiple circumferential crack initiations at the canaliculi. A single canaliculus could initiate more than one crack (double arrow). Osteocyte lacunae are identified by asterisks; (c) Views (xy, xz, and yz) of a single crack associated with a canaliculus (arrowhead). Dashed lines are approximated lamellar boundaries; (d) Merging process of the circumferential cracks in the circumferential and depth (z-plane) directions; (e) Bright and dark (arrowheads) layers damage associated with the formation of a shear band. The microcracks have a "sheet-like" appearance through the depth (z-plane). Note also the radially oriented microcrack at the Haversian canal (empty arrowhead). Compressive load applied horizontally for b-d-e and obliquely for c.

Fig. 5. Physical interpretation of sub-lamellar structure - microcracking relations in bone under transverse compression. (a) Schematic of multiple circumferential crack nucleations and mergings at the radially oriented canaliculi (x-direction) under interlamellar shear (stress state depicted) in osteonal layers with fibrils oriented parallel to the osteons; (b) Proposed interfibrillar nature of the sub-lamellar cracks (light grey) with respect to the rotating mineralized collagen fibrils (minerals in black) and the surrounding non-collagenous proteins (small grey lines). Arrows indicate the stress state depicted in (a).