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Review

# Bone hierarchical structure in three dimensions ☆

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ABSTRACT

Bone is a complex hierarchically structured family of materials that includes a network of cells and their interconnected cell processes. New insights into the 3-D structure of various bone materials (mainly rat and human lamellar bone and minipig fibrolamellar bone) were obtained using a focused ion beam electron microscope and the serial surface view method. These studies revealed the presence of two different materials, the major material being the well-known ordered arrays of mineralized collagen fibrils and associated macromolecules, and the minor component being a relatively disordered material composed of individual collagen fibrils with no preferred orientation, with crystals inside and possibly between fibrils, and extensive ground mass. Significantly, the canaliculi and their cell processes are confined within the disordered material. Here we present a new hierarchical scheme for several bone tissue types that incorporates these two materials. The new scheme updates the hierarchical scheme presented by Weiner and Wagner (1998). We discuss the structures at different hierarchical levels with the aim of obtaining further insights into structure–function-related questions, as well as defining some remaining unanswered questions.



## 1. Introduction

The material bone is present in a family of mineralized connective tissues that share some common structural characteristics and fulfil diverse mechanical functions. A characteristic elementary unit of bone is the mineralized collagen fibril. The mineralized fibril, together with non-collagenous proteins and water, arranged in a complex hierarchical structure, are ultimately responsible for the mechanical properties of the material [1–3]. Although categorizing a material such as bone into hierarchical levels of organization is a somewhat arbitrary exercise, such a conceptual understanding of the structure is important when addressing fundamental questions, such as how cells build this complex material, what aspects of the structure are responsible for different mechanical, metabolic and sensing functions, and how changes in the structure (pathologic or physiologic) affect its mechanical function.

The complex structure of bone and its relation to function has been the subject of investigation for more than 320 years, and is still far from being well understood. For the first 200 years or so, the main tool for probing the structure of bone was the light microscope. In fact, bone was one of the first materials to be examined with the development of "modern" microscopes at the end of the seventeenth century. These studies identified what we know today are resorption cavities, lacunae and osteons (Haversian systems) [4], as well as lamellae [5]. Spongy bone was described by Munro [6]. The development of the polarizing microscope in the second half of the nineteenth century enabled investigators to distinguish between various functional members of the bone family of materials. The first detailed description of osteons as a prominent component of compact bone is by Gebhardt [7]. Gebhardt described alternating orientations of fibrils in subsequent lamellar layers of an osteon and noted that the angular offset between adjacent layers might be steep or shallow. The different bone types that we recognize today (woven, lamellar, fibrolamellar, etc.) were described by Weidenreich [8]. For an excellent review of the history of bone research see Ref. [9].

The mineral phase of bone also has a long history of investigation. Calcium, phosphate and carbonate were identified as the major ionic constituents of bone mineral in 1799 [10], their relative proportions in 1858 [11]. The mineral itself was identified as hydroxyapatite by X-ray diffraction in 1926 [12]. As the mineral phase contains significant amounts of carbonate, it is differentiated from hydroxyapatite and is called carbonate hydroxyapatite (or dahllite) [13]. Polarized light and X-ray diffraction were used to show that the *c*-axes of the crystals are aligned with the collagen fiber axes [14,15]. Robinson was the first to use the transmission

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electron microscope to study bone. He showed that the crystals were plate-shaped [16] and documented the orderly organization of the crystals within the type I collagen structure [17]. The close association of crystals and collagen was further explored [18], and finally the structural basis for this distribution was elucidated when Hodge and Petruska [19] identified the staggered array model for the type I collagen structure. This resulted in models of the 2-D distribution of the crystals, which was preferentially in the gap zones as compared to the overlap zones. However, the 3-D organization in which plate-shaped crystals are arranged in layers that span the collagen fibril was only identified much later [20].

Around two decades ago, Weiner and Wagner [1] described the then-current state of knowledge regarding the architecture of the different hierarchies of lamellar bone. During the last few years we have employed a novel structural analysis method that has allowed us to elucidate the organization of bone at 10 nm resolution in three dimensions. This has enabled us to gain further insights into bone hierarchical organization, which in turn has motivated us to update the hierarchical structural scheme presented by Weiner and Wagner [1].

The 3-D structural determination method that we used was described by Heymann et al. [21] and exploits the capability of the focused ion beam (FIB) scanning electron microscope (SEM) to sequentially cut thin (ca. 10 nm) slices off an embedded block and image the block face. The method is called "slice and view" or "serial surface view" (SSV) [22]. We first adapted the method to study the collagenous matrix of demineralized rat circumferential lamellar bone [23]. The SSV method has since been applied to demineralized horse osteonal lamellar bone [24], human lamellar bone [25], minipig fibrolamellar bone [26], human trabecular bone (in preparation) and lamellated fish bone (in preparation).

The most fundamental observation at the microscale revealed by SSV is the discovery that lamellar bone is essentially composed of two different materials: a predominant ordered material and a disordered material. The ordered material is composed of aligned arrays of mineralized collagen fibrils arranged in different structural patterns (essentially following Gebhardt [7] and many subsequent studies), and the disordered material is composed of a mineralized matrix with poorly oriented individual type I collagen fibrils and abundant non-collagenous organic material. Most significantly, the cellular components of bone, namely the osteocyte cell processes housed in the canaliculi, are confined to the disordered material [25]. In 1947, Ruth [27] provided a remarkably good description of the disordered material in human bone, which he referred to as the "diffuse lamellae". "The diffuse lamellae are bands of radially oriented fibrillae, loosely disposed, and separated from each other by relatively wider interfibrillar spaces filled with granular substance. The fibrillae themselves are delicate strands disposed at right angles to the compact lamellae" [27] (page 44). Fig. 1 shows pairs of SSV images of ordered and disordered materials (see also SV1), and Fig. 2 is a schematic based on the human bone study showing that the disordered material is a continuous phase that essentially fills in the spaces between the ordered fibril arrays [25]. The rough proportions of ordered and disordered materials in human bone are 4:1. This was calculated volumetrically from the SSV slices showing ordered and disordered collagen arrays.

Much still remains to be learned about these two materials in lamellar bone and other types of bone, as well as from different bones within the same species and from different species.

## 2. Hierarchical organization of bone in three dimensions

The starting point for the hierarchical scheme presented here is the proposal by Weiner and Wagner [1] that lamellar bone can be viewed as having seven levels of hierarchical organization. We adapt the Weiner and Wagner scheme to take into account the presence of the ordered and disordered materials, as well as some insights gained using SSV for higher-level organization of other bone types. The new scheme (Fig. 3) is also relevant mainly to lamellar bone, as this is by far the most common structural type found in bones [28]. Fig. 4 is basically the same scheme, but where the components are mainly illustrated with micrographs.

The schemes in Figs. 3 and 4 arbitrarily divide bone into nine hierarchical levels. This really applies only to lamellar bone. Some members of the bone family of materials could well be divided into fewer hierarchical levels, such as woven bone. More work is certainly needed to confirm the presence of both ordered and disordered materials in other bone types. We also assume that levels I, II and III are common to all bone types, and here again this should be examined further. Below we discuss each level separately following the schemes in Figs. 3 and 4.

### 2.1. Level I: the major components

The biomolecular and mineral components of both the ordered and disordered materials are similar (at this coarse level of description). The ordered material comprises mainly the mineral carbonated hydroxyapatite, type I collagen and water, with minor amounts of other collagen types, non-collagenous proteins (NCPs) and proteoglycans (GAGs) [29]. The disordered material is composed mainly of carbonated hydroxyapatite, type I collagen, and, based on the prevalence of what can be loosely termed "ground mass", we infer that relatively large amounts of non-collagenous proteins, proteoglycans and water are also present. Much more remains to be discovered regarding the specific molecular components of the disordered material.

#### 2.1.1. Mineral phase

The mineral phase of mature bone is carbonated hydroxyapatite in the form of thin (1.5–4 nm thick, Fig. 5) plate-shaped crystals [16,30,31]. The mature carbonated hydroxyapatite crystals are relatively disordered at the atomic level, in part because the crystals form from a highly disordered precursor phase [32], have many included additives (such as carbonate) [13,33] and the mature crystals are very thin [31] and hence have a large surface to bulk ratios [34]. The crystal surface is known to be relatively disordered compared to the bulk [35].

#### 2.1.2. Collagen

Type I collagen is the most abundant protein in mature bone. Other collagen types, including types III, VI and V, are also present [36,37]. The staggered array structure of the triple helical molecules of type I collagen [19] results in the formation of spaces (often referred to as holes) within the fibril [38]. In bone these holes are aligned to form thin (around 1.5 nm thick) extended slots (called grooves) in which the intrafibrillar crystals form [1,39]. Based on the presence of the characteristic 67 nm repeat structure in the collagen fibrils from both the ordered and disordered materials, we assume that type I collagen is a major component of both the ordered and the disordered materials.

#### 2.1.3. Non-collagenous proteins (NCPs) and proteoglycans

There are many non-collagenous proteins in bone, but most of them are not unique to bone. There are, however, several bone-related proteoglycans (such as biglycan and decorin) and a series of bone-related non-collagenous proteins (such as osteocalcin, matrix Gla protein, osteonectin, alkaline phosphatase, BAG-75 and RGD-containing proteins, including osteopontin and bone sialoprotein) that are thought to play crucial roles in bone formation [40]. We know surprisingly little about the precise locations

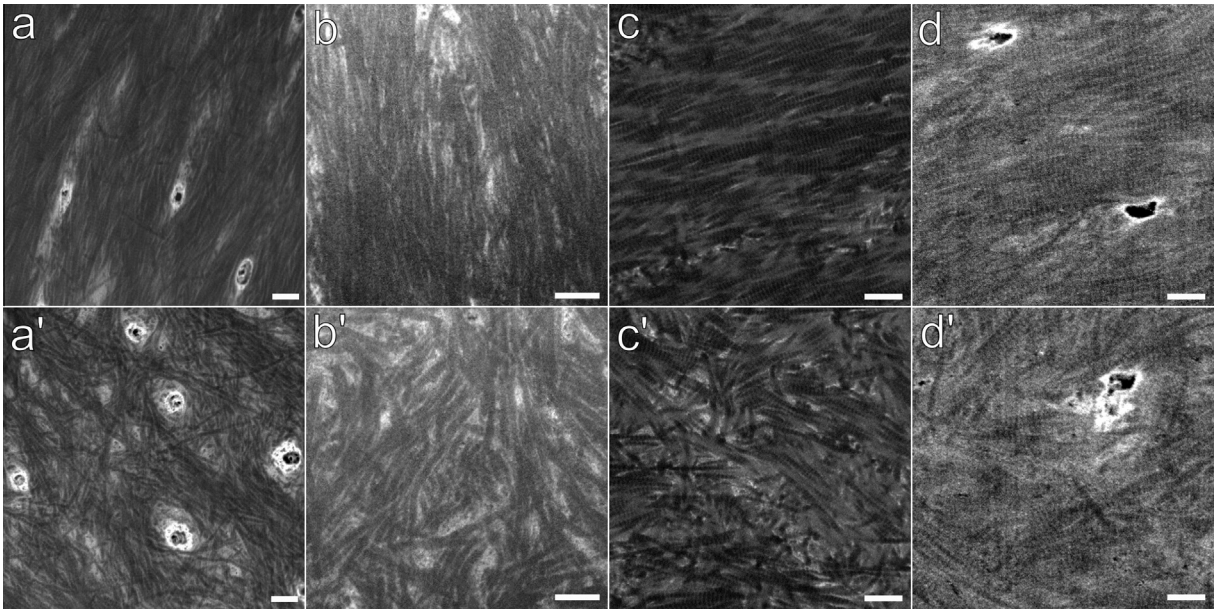


**Fig. 1.** Selected images of ordered and disordered materials in lamellar bone from SSV stacks. Four pairs of images were taken from rat circumferential lamellar bone (a, a′), human cortical osteonal (b, b′) and circumferential lamellar bone (c, c′), and human trabecular lamellar bone (d, d′). Each pair was selected from one continuous SSV volume and the two images in each pair are separated by 0.5–1.0 microns. Scale bars: 1 micron.

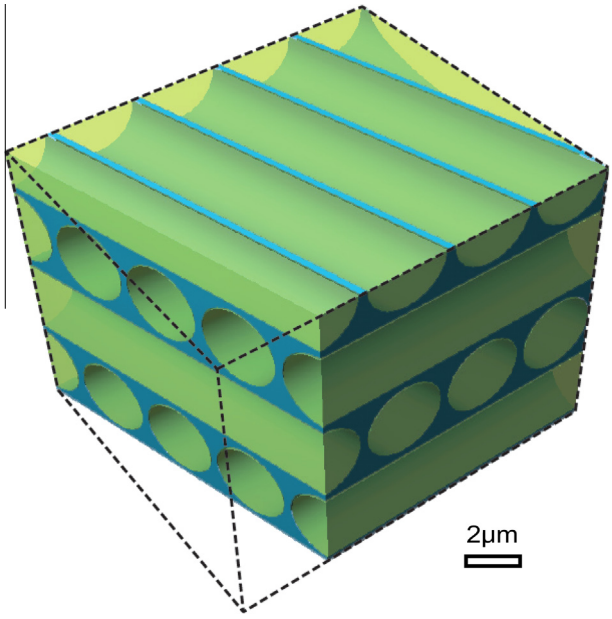


**Fig. 2.** Schematic drawing of human lamellar bone, showing differently oriented collagen fibril bundles (green). The narrow spaces between the bundles and thicker spaces between adjacent layers of bundles are filled with the disordered material (blue). Note that the bundles of adjacent layers depicted are not orthogonal. Adapted from Ref. [25] and reproduced with permission.

of specific NCP macromolecules in bone or their specific functions. It would be interesting to determine which of these macromolecules are located in the disordered material, which are in the ordered material and which are in both materials.

*2.1.4. Water*

Water is an essential component of bone. The various types of water in bone should also be integrated into the different hierarchical organizational levels. At level I (Fig. 3), the level of the crystals, water is known to be bound to the crystal surface [41]. This is particularly prevalent when the crystal surface is composed of a disordered amorphous calcium phosphate (ACP)-like layer [35]. Other water molecules are present between the collagen triple helical molecules when mineral is absent or only partially present, accounting for the closer spacing of these molecules upon drying [42]. When lamellar bone is dehydrated, the lamellae contract more in the direction perpendicular to the lamellar boundary than in the orthogonal direction (level VI in Fig. 3). This indicates that, even in mineralized bone, liquid water is present in the collagen channels [43]. Unbound water is presumably present in the canaliculi, lacunae and blood vessels. For an overview of water in bone and in mineralization, see Ref. [44].

*2.2. Level II: structural components*

The collagen molecules are organized into fibrils of some 80–120 nm in diameter, which may well be oval shaped [45]. Crystals of carbonated hydroxyapatite nucleate from a disordered precursor phase [32,46] within the gaps inside the fibril [47,48], and with growth extend into the overlap zones [49]. The end product is the mineralized collagen fibril that comprises layers of plate-shaped crystals that span the cross-section of the fibril [20,50]. The fibril is therefore not radially symmetrical, but has an orthotropic, essentially crystalline, structure (Fig. 6).

Mineralized collagen fibrils are the major component of the ordered material. The *c*-axes of the crystals are well aligned with the collagen fibril axis [14,15]. The disordered material also contains mineralized collagen fibrils, which are only one of the major constituents, together with the abundant ground mass and the canaliculi. In the ordered material, most of the crystals are intimately associated with the collagen fibrils (in the interior or on the surface). In the disordered material, Reznikov et al. [25] observed crystals both within the collagen fibrils and between them. The textures of these disordered material images are very similar to those reported by others [51,52], who also came to the conclusion that crystals are located both within and between fibrils. They did not, however, know about the existence of the disordered material, and therefore proposed that this intrafibrillar and extrafibrillar crystal motif was characteristic of all lamellar bone.

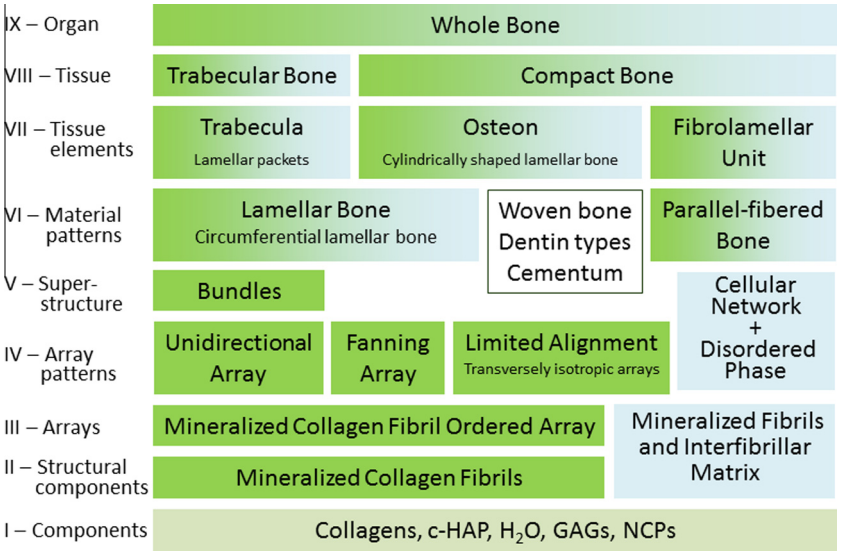


**Fig. 3.** Scheme showing the hierarchical organization of bone. Up to level V the hierarchical levels can be divided into the ordered material (green) and the disordered material (blue). At level VI, these two materials combine in lamellar bone [25] and parallel fibered bone [26]. Other members of the bone family still need to be investigated with respect to the presence of both material types, hence they are depicted in a box without color. Level VII depicts the lamellar packets that make up trabecular bone material and the cylindrically shaped lamellar bone that makes up osteonal bone. The fibrolamellar unit comprises the primary hypercalcified layer, parallel fibered bone and lamellar bone [26]. c-HAP: carbonated hydroxyapatite; GAGs: glycosaminoglycans; NCPs: non-collagenous proteins.

### 2.3. Level III: arrays

Type I collagen fibrils have a strong tendency to self-assemble into arrays, at least in vitro, with the fibril long axes aligned [53]. The assembly process in vivo is complex, apparently starting in the endoplasmic reticulum of the osteoblasts and continuing in compartments outside the cytoplasm, and finally extending into the extracellular space [54,55]. The diameters of these arrays can vary between less than a micron to several microns. Fibril arrays are present only in the ordered material (SV1). In the disordered material the majority of fibrils appear as individual fibrils and show little preferred orientation compared to the ordered material (see the histograms in Fig. 4, and also Fig. 1) [25].

### 2.4. Levels IV: array patterns

The fibril arrays may be organized in a variety of ways to form different patterns [1,56]. In the ordered material this is effectively the first hierarchical level at which differences in structure appear between different members of the family of bone materials, and even within the same bone type. The simplest and most anisotropic pattern is essentially an extension of the single array with all the fibril axes aligned to much larger dimensions (Fig. 7). This so-called unidirectional array pattern is common in lamellar bone [57], parallel fibered bone [26], Sharpey's fibers, mineralized tendon and other bone types [58].

A second pattern is the fanning array, in which the orientations of the fibril array change in a gradational manner [23] (Fig. 8). This structural pattern has also been called twisted plywood, and is common in lamellar bone [59]. It has been observed that fanning/twisted arrays can form in vitro when the collagen concentrations are relatively high [53].

The disordered material can be found as thin layers between the aligned collagen fibril arrays (see Figs. 7 and 8 and SV1), but can also be found as relatively extensive layers. In these layers the collagen fibrils are generally present not as arrays but as individual fibrils, and have no preferred orientation in three dimensions. The spaces between the collagen fibrils are filled with ground mass (Fig. 9). This pattern is reminiscent of woven bone [56], but differs from woven bone in that the fibrils exist mostly as individuals and not as randomly oriented arrays [25].

### 2.5. Level V: super-structure

The SSV 3-D structures of both human lamellar bone and minipig fibrolamellar bone (but not rat lamellar bone) reveal the presence of unidirectional collagen fibril bundles in the ordered material (Fig. 1). These bundles are usually 1–3 microns in diameter. Within the bundle neighboring fibrils are mostly in register with respect to the 67 nm repeating structure, and the packing of the fibrils in the bundles is tight and space filling. The fibrils thus form a highly anisotropic and co-oriented array (Fig. 10). These cylindrical arrays are aligned side-by-side (Fig. 2) to form extensive sheets of parallel bundles. The sheets of differently oriented bundles stack in such a way that every other set of parallel bundles has a similar orientation. The angular offset between adjacent sets of co-aligned bundles varies between 40 and 80°. We have never documented a canonical orthogonal organization of bundles.

The bundles do, however, also contain small amounts of ground mass. The treatment of demineralized bone with Alcian Blue stain for staining chondroitin sulfate results in the appearance of unusual "hour-glass structures" staggered within individual bundles [25]. These hour-glass structures may reflect an intimate association between ordered collagen fibril arrays and proteoglycans (see Fig. 5 in Ref. [25]).

In human bone the bundles are separated by thin layers of disordered material, and these thin layers often include the osteocyte processes (Fig. 1) [25]. In fibrolamellar bone there are two types of bundles: those bounded by sheaths of unknown composition and those like in human bone, by the thin layers of disordered material [26]. Much remains to be learned about these bundles in terms of their structure, composition and function. Silver et al. [55]also observed fiber bundles in tendon, and showed that their presence is intimately involved in the intracellular synthesis and assembly of collagen. We suspect that the presence of bundles in some bones also reflects the manner in which the cells produce, package and export collagen fibrils. We therefore include a special hierarchical level for collagen bundles.

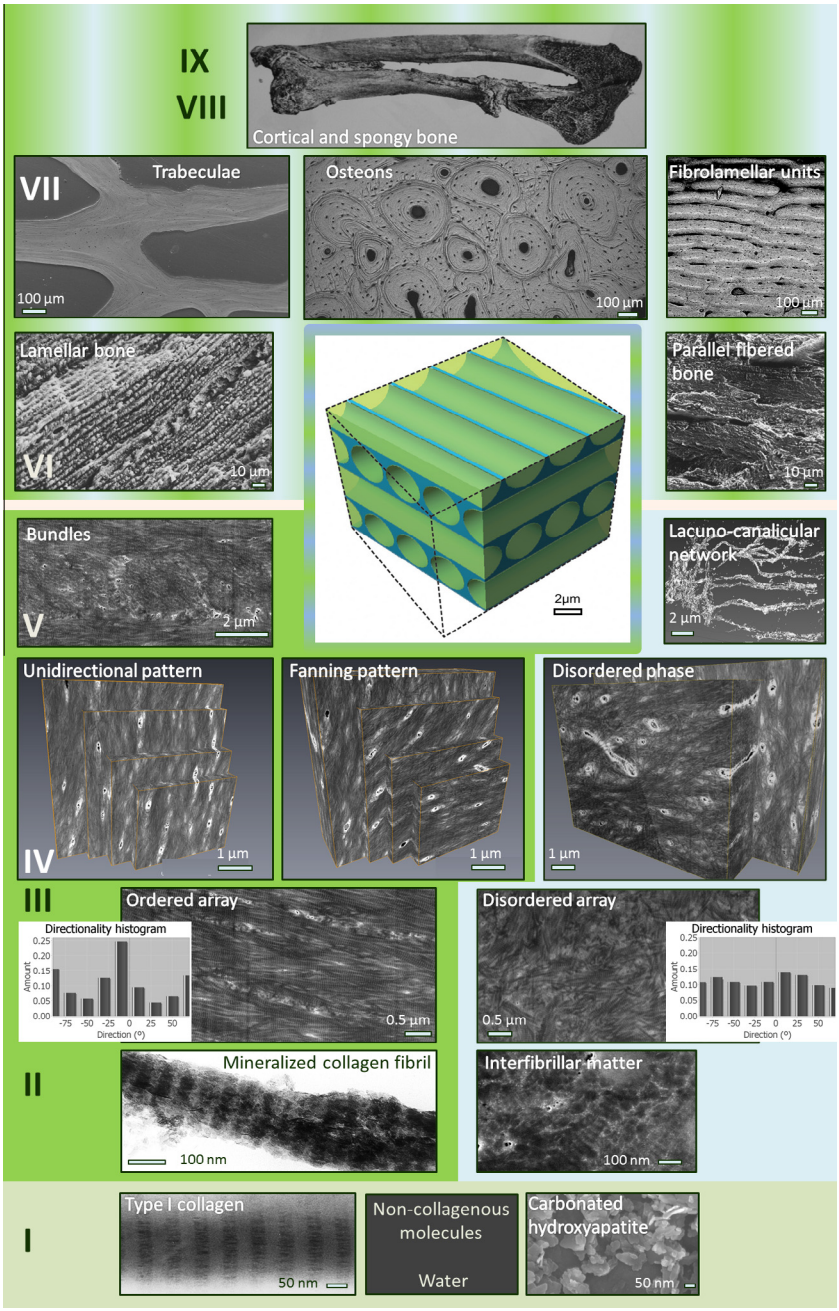


**Fig. 4.** Essentially the same scheme as shown in Fig. 3, but mainly illustrated with representative images of the different hierarchical levels. Many of these images are reproduced in the text below and explained in more detail. The schematic illustration is adapted from Reznikov et al. [25]. The borders of the images are color coded as in Fig. 3: green for the ordered material, blue for the disordered material and a graded color scale where both materials are present. The schematic is also colored in the same way. The histograms at level III show the extent of alignment of the collagen fibrils in the ordered and the disordered materials. The whole bone image depicting levels VIII and IX is reproduced from Julius Wolff [29].

Note that the term "bundle" is used fairly frequently in the bone literature. Weidenreich [8] classified bone types according to bundles with varying properties (see Ref. [56]). There is a bone structure type in alveolar bone called "bundle bone" [60], but this appears to resemble the mineralized insertions of collagen fibers from the periodontal ligament.

In the continuous disordered material, the super-structure is the combination of the material itself (mineralized collagen fibrils and ground mass) and the embedded osteocyte lacunae and canaliculi, which are confined within the disordered material (SV1). The canaliculi and the lacunae contain the processes extending out from the osteocytes and the osteocytes themselves, respectively. There is thus an intimate structural relationship between the organization of the osteocyte network and the collagen matrix arrangements [61]. The disordered material fills in the spaces between bundles in human bone (Fig. 2), and thus the 3-D geometry of the canaliculi also reflects the overall distribution of the bundles. During bone formation, it could well be that the locations of the cell processes and their surrounding disordered material define the spaces in which the ordered material is deposited. Fig. 11 shows the 3-D organization of the canalicular network and part of an osteocyte in human osteonal bone.

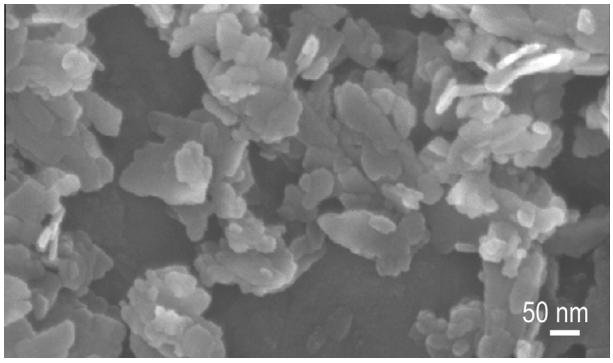


**Fig. 5.** Scanning electron microscope image of plate-shaped crystals of bone carbonate hydroxyapatite.

### 2.6. Level VI: material patterns

At this level we refer to the materials/tissues that make up an object such as a bone or a tooth. As these materials are the products of cellular activities and some also include cell components, they are also referred to as tissues. The materials/tissues in a tooth that belong to the bone family of materials are mainly intertubular dentin and cementum. The most common material that makes up a bone is lamellar bone. Fibrolamellar bone (also known as plexiform bone) is common in many rapidly developing animals [62]. The major material components of fibrolamellar bone are parallel fibered bone and lamellar bone [26,63]. Woven bone constitutes a material in its own right [56,62]. We confine the discussion here to the materials/tissue types in bones (as opposed to teeth).

#### 2.6.1. Woven bone

The term "woven bone" is used in different ways (compare Pritchard [56] to Currey [64]). Following Su et al. [65], we regard woven bone as being composed of mineralized collagen fibril bundles that have little or no preferred orientation in three dimensions. Woven bone may not have any higher hierarchical levels of organization. Woven bone is a transient tissue type that is frequently deposited during development, as well as fracture repair [62]. Woven bone material seems to represents an optimal solution for cases where a scaffold is needed for subsequent formation of more structured bone (as in bone development) or for the early stages of callus formation during fracture healing. This is due to the relative speed with which it can be laid down, and the similarity of its mechanical properties in all directions. Much more needs to be understood about woven bone's 3-D structure.

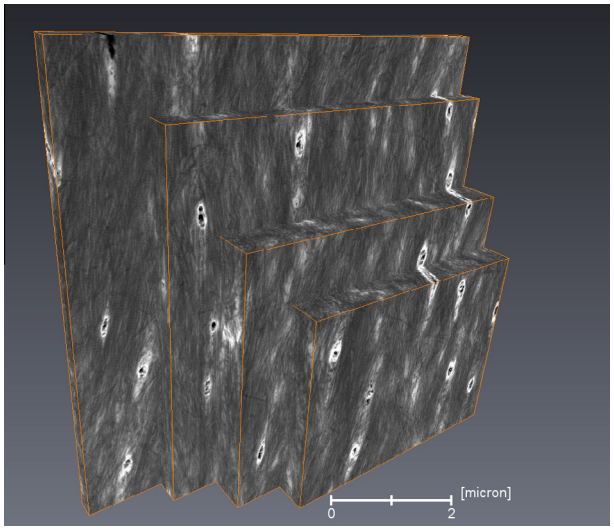


**Fig. 7.** Reconstruction of the unidirectional array using images obtained from an SSV stack from rat lamellar bone [23]. Note that most of the collagen fibrils with their characteristic 67 nm banding pattern are aligned in one direction over a thickness of about 2 microns. The disordered material is locally present in the form of thin layers embedded within the arrays, and the canaliculi (round holes surrounded by bright highly staining material) are confined within the disordered material. The reconstruction is reproduced from Fig. 4.

#### 2.6.2. Parallel fibered bone

Parallel fibered bone is composed mainly of unidirectional ordered bundles of mineralized collagen fibrils [66]. These bundles are separated from each other by the thin layers of disordered material. Parallel fibered bone is the major constituent of fibrolamellar (also known as plexiform) bone [26]. It is also the organizational motif of mineralized tendons and Sharpey's fibers [1]. Fig. 12 shows a fracture surface of bovine fibrolamellar bone, in which the major component is parallel fibered bone. The parallel fibered bone is flanked by lamellar bone.

#### 2.6.3. Lamellar bone

Lamellar bone is composed of a series of lamellae, each lamella contains both the ordered material and the disordered material with its embedded canaliculi [25]. The thicknesses of individual lamellae range from 3 to 7 microns. Each lamella contains differently oriented bundles of both unidirectional arrays of mineralized fibrils and fanning arrays, and the spaces between these bundles are filled by the disordered material (Figs. 1 and 2) [25]. The canaliculi embedded in the disordered material are generally aligned perpendicular to the lamellar boundary plane. It has been proposed

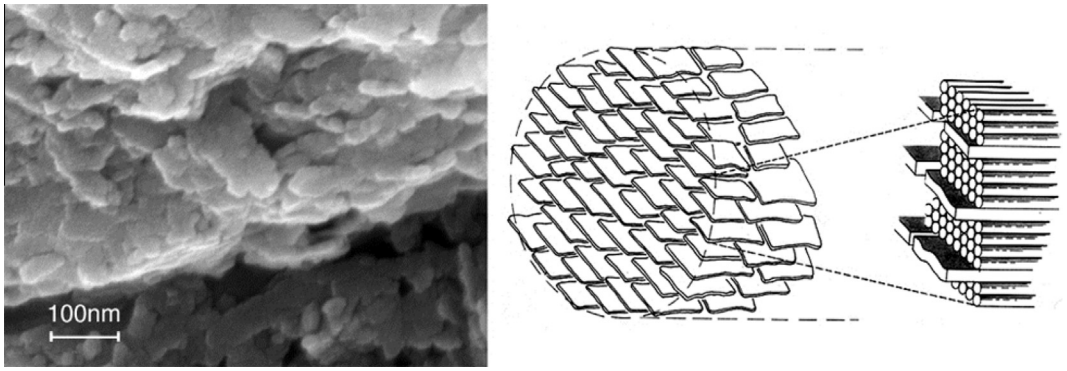


**Fig. 6.** SEM image of a fracture surface of baboon lamellar bone (reproduced from Ref. [52]) showing the plate shaped crystals arranged in layers. This crystal organization pattern, first identified by Weiner and Traub in 1986 [20], is shown schematically and related to the collagen structure.

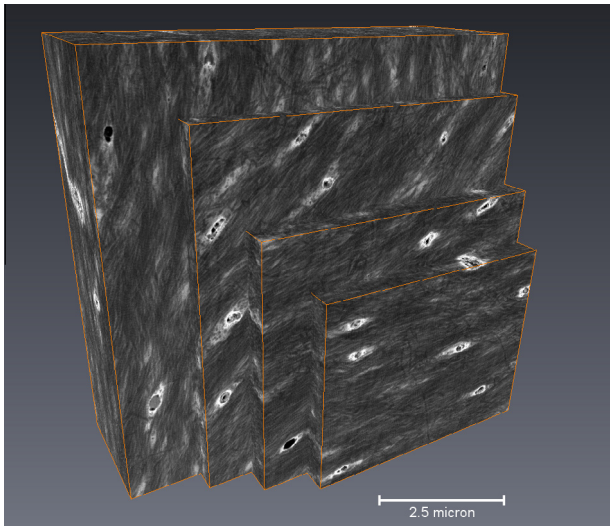


**Fig. 8.** Reconstruction of the fanning array using images obtained from an SSV stack from rat lamellar bone [23]. Note how the directions of the aligned collagen fibrils change progressively. The thin layers of disordered material are embedded inside the fibril arrays. The reconstruction is reproduced from Fig. 4.

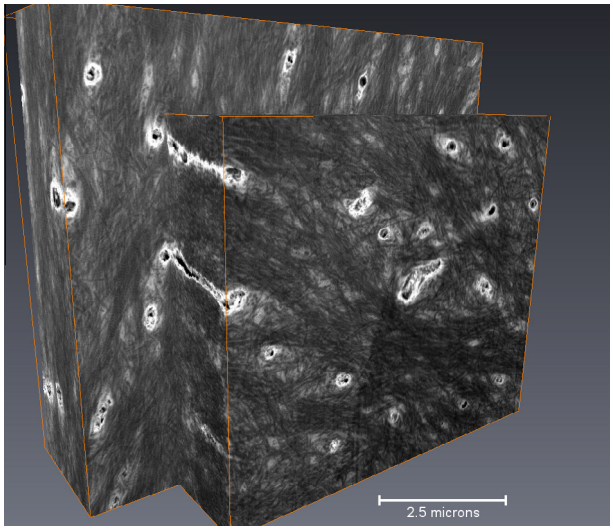


**Fig. 9.** Reconstruction showing an extensive layer of disordered material in front of a layer of aligned fibril arrays. These extensive layers of disordered material are generally located at the interphase between two aligned layers that have different preferred orientations. The reconstruction is reproduced from Fig. 4.

that the layers of crystals within an individual lamella have different orientations (the so-called rotated plywood model) [45].

Circumferential lamellar bone (CLB) comprises a series of lamellae with a large radius of curvature that are all parallel to the forming surfaces of both compact and trabecular bone (Figs. 4 and 13). The CLB lamellae are laid down by the osteoblasts on the bone surface. This primary bone type is replaced by secondary osteonal bone (especially in large animals) (level VII in our scheme), and thus the presence of CLB on a bone surface indicates that this specific locus has not been remodeled. A comparison of the 3-D structures of CLB and osteonal bone in the human femur did not reveal any significant differences at the fibril organization level [25].

### 2.7. Level VII: tissue elements

Lamellar bone is by far the most common structural element in mammalian bone [28]. Furthermore, during the process of remodeling, lamellar bone replaces woven bone and fibrolamellar bone. The continued description of hierarchical levels of organization is therefore confined to lamellar bone elements. Depending upon when and where the bone forms, the lamellae adopt several different structural motifs in addition to the circumferential lamellar motif (level VI).

#### 2.7.1. Lamellar packets

The term "lamellar packets" is used to refer to an assemblage of slightly differently oriented series of lamellae that are characteristically found in trabecular bone (also called spongy bone) [67] (Fig. 4). Adjacent series truncate each other at low angles (Fig. 14). This motif is formed as a result of the removal of some lamellar bone, followed by the deposition of new lamellar bone to fill in the resulting resorption defect. Thus the most superficially deposited lamellar packet is aligned with the surface of the spongy bone strut but is not necessarily aligned with the earlier surface, reflecting the adaptational history [54].

#### 2.7.2. Osteons

Osteons are often classified as primary or secondary osteons, and the latter are also referred to as Haversian systems. Secondary osteons are the products of bone remodeling and are most abundant in mature skeletons, particularly of large animals [62]. Primary osteons have the same concentric lamellar structure but they do not have a cement line, the outer layer of the secondary osteon. The cement line forms where resorption ceased and new lamellae started being laid down. Primary osteons form de novo around blood vessels such that the pre-existing cavity is centripetally filled with lamellar bone. Primary osteons are common in fibrolamellar bone when a vascular cavity is being filled in [62] and at the interface of compact and trabecular bone of large animals (authors' observation).

The secondary osteons, or Haversian systems, are roughly cylindrical structures about 100–200 microns in diameter with a central canal of some 30–40 microns diameter (Fig. 4). The central canals may branch or merge and are often co-aligned in long bones, with the prevailing direction being along the bone axis. Historically, the longitudinal Haversian canals were distinguished from the transverse Volkmann's canals. However, high-resolution microcomputerized tomographic reconstructions show the continuity of these canals and indicate that they are parts of the same system [68,69]. Gebhardt [7] described alternating orientations of fibrils in adjacent lamellae of an osteon and noted that the angular offset varies. It follows from Gebhardt's study that none of the lamellar layers in an osteon contain collagen fibrils oriented strictly parallel or strictly perpendicular to the cylinder axis. Therefore, following Gebhardt, the lamellae that form an osteon could be viewed as a set of nested "coil springs" with alternating pitches [70]. The average pitch of lamellae may differ between osteons, resulting in the characteristic appearance of dark and bright osteons in the polarized-light microscope. This phenomenon was extensively studied from the viewpoint of local adaptation of cortical bone to the dominant mode of loading [71–73].

Secondary (but not primary) osteons are surrounded by cement lines [62]. In trabecular bone, each aligned series of lamellae within the lamellar packet is also bound by a cement line [67]. The cement line is thinner than an individual lamella and appears crenulated [62,74]. The material deposited in the cement line and its structure are still poorly understood [74–76]. Although some controversy still exists regarding its degree of mineralization, the prevailing view is that it is more highly mineralized than the associated lamellar bone [74].

#### 2.7.3. Fibrolamellar bone

Fibrolamellar bone is composed of repeating units that form sequentially [62]. After the vascular system is in place, the first

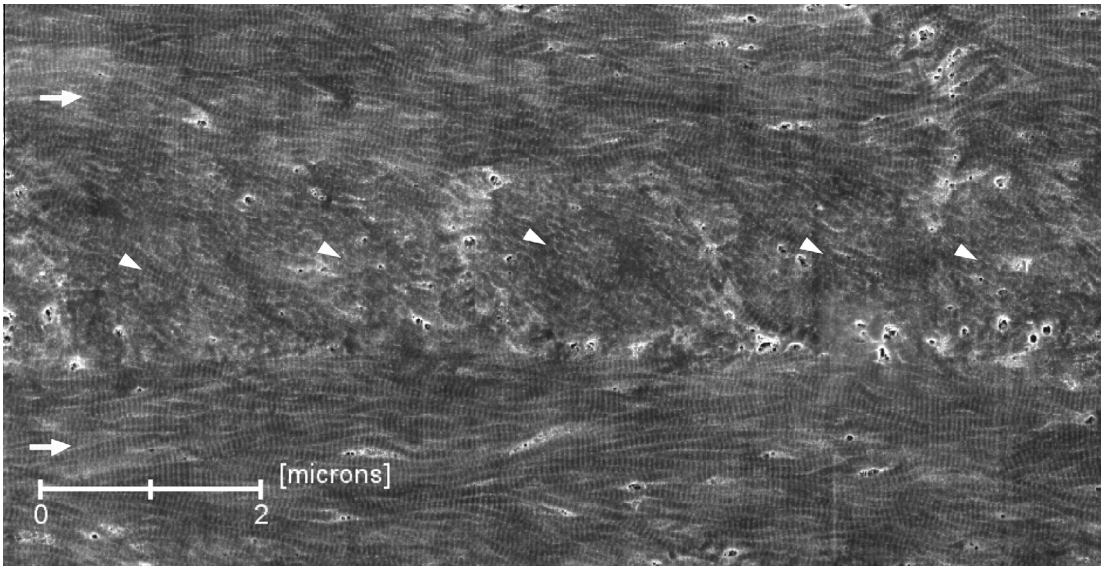


**Fig. 10.** Image from a 3-D volume of human lamellar bone showing bundles in cross-section (arrowheads) and bundles in the plane of the image (arrows). The plane of the image is oriented perpendicular to the lamellar boundaries. The collagen fibril D-spacings are clearly visible.

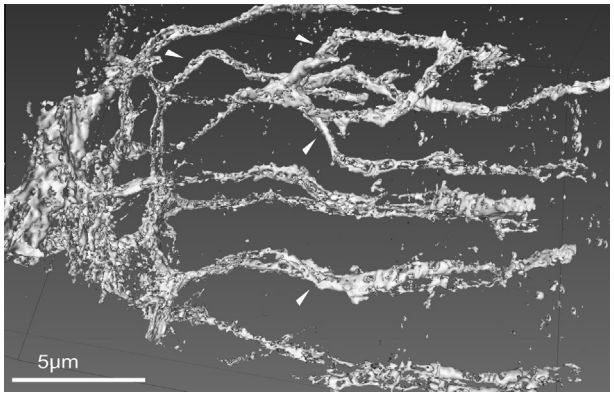


**Fig. 11.** Reconstruction of part of an osteocyte and its associated canaliculi in human osteonal bone. Note that the kinks in the canaliculi (arrowheads) often occur when the bundles of ordered collagen fibril arrays (not visible in this image) change direction.

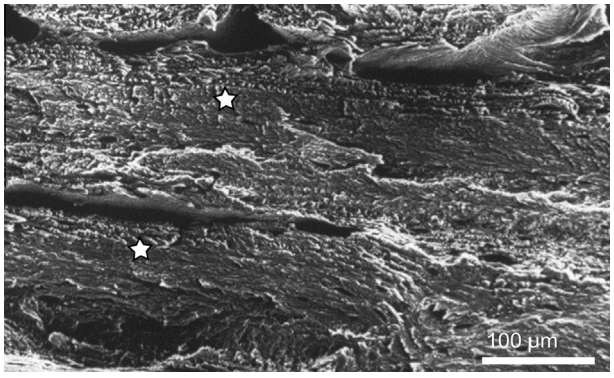


**Fig. 12.** SEM image of a transverse fracture surface of an immature bovine long bone. Parallel-fibered bone tissue (asterisks) constitutes the bulk of the elongated horizontal formations (the fibrolamellar units, see below).

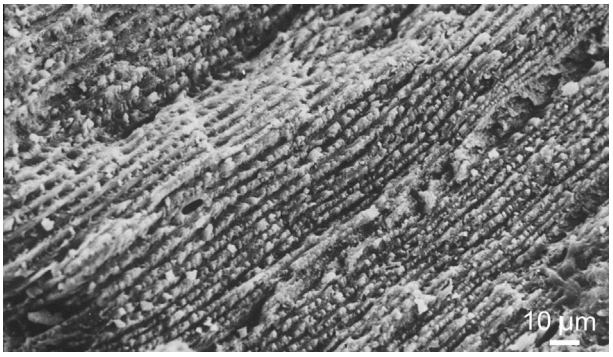


**Fig. 13.** SEM image of a transverse fracture surface of a rat long bone showing a vast area of aligned lamellae.

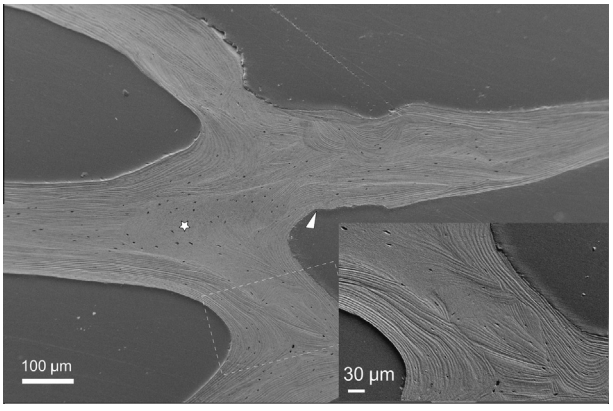


**Fig. 14.** SEM image of a polished embedded section of trabecular bone from a human proximal femur. Note the patchy appearance of overlapping lamellar packets. The last formed packets have lamellae parallel to the surface. The older lamellar packets are misaligned with the last formed lamellar packets. The featureless area in the center of a strut (asterisk) is presumably a fragment of the primary spongiosa. The discontinuity of a surface lamellar packet (arrowhead) indicates a resorption event. The inset shows lamellar packet arrangements in a single strut of trabecular bone at higher magnification.

material deposited is the primary hypercalcified layer, consisting of mineralized collagen fibril bundles with no preferred orientation, large amounts of intervening ground mass and many small pores [26]. The next step is the deposition of thick layers of parallel fibered bone on both sides of the primary hypercalcified layer. Finally, the remaining voids are filled in with lamellar bone [62]. The vast majority of fibrils in the parallel fibered and lamellar bone components are aligned with the long axis of the bone, making this a highly anisotropic structure. Fibrolamellar bone also has a disordered material that fills in the spaces between the collagen bundles, and the canalicular network is embedded within this disordered material [26].

### 2.8. Levels VIII and IX: tissue and the organ (whole bone)

All bones contain an outer shell of compact bone, which can vary greatly in thickness even within an individual bone. Trabecular bone (also known as spongy or cancellous bone) may fill up the entire inner volume of some bones, such as vertebrae, ribs and calvarial bones, or may only be present in parts of bones, such as the epiphyses of long bones.

Structurally both compact and trabecular bone are composed of lamellae, and a comparison of the 3-D structures up to level VI in Figs. 3 and 4 (for human bones) reveals only subtle differences. One such difference is that in compact bone from human femora repeating sets of unidirectional fibril bundles show a variety of alternating orientations, whereas in a trabecular bone lamella one of the two unidirectional fibril sets is more or less aligned with the long axis of the individual trabecular strut (Reznikov et al., in preparation). Another difference is that, on average, trabecular bone material contains less mineral than the associated compact bone [62].

Probably the most intensively investigated structural attribute of bone is at level VII, namely whether or not the overall texture of trabecular bone reflects the predominant stress directions to which the whole bone is subjected. Julius Wolff was one of the first to propose that indeed it does [77], and D'Arcy Thompson is one of the most well known proponents of this idea [78]. There are several compelling studies that, in our opinion, support the notion that there is a relationship between the applied stress field and trabecular texture [79], including two experimental studies in which the mode of the applied stress field was changed and the trabecular orientation changed accordingly [80,81].

## 3. Discussion

Our understanding of the hierarchical structure of bone will always be a "work in progress" as long as new structural information continues to become available. A retrospective view of the 300 or so years of investigation of bone structure shows that new insights were often obtained when new technologies were developed. The recent use of the FIB SEM and the SSV methodology have led to the discovery (in the relatively few bones investigated so far) that lamellar bone as well as fibrolamellar bone is composed of two different materials – the disordered and ordered materials. Other members of the bone family of materials still need to be examined in this regard. Furthermore, this technology enables bone collagen fibril organization to be analyzed quantitatively, and thus opens the way to assessing many fundamental questions regarding subtle structure–function relations. In the following brief discussion, we focus on identifying open questions that arise from the structural analysis presented above.

### 3.1. Structural issues

The capability of analyzing the 3-D structure of bone at high resolution (around 10 nm), in a relatively large volume (tens of microns) and carrying out detailed quantitative analyses of each of the hundreds of images in a single stack opens up the possibility of addressing some fundamental issues in bone structure. One such issue, for example, is whether the lamellar structures of compact and trabecular bone within a single bone are different. Our study of human femora confirmed the study of Ruth [27], showing that they are essentially the same within the inherent range of structural variability observed in both cases [25]. Another question is whether the lamellar structures in intra-membranous bones and endochondral bones are different. This might reflect the modes of formation of these bones and/or the different functions that they perform. Does the lamellar structure change significantly when the bone's mechanical function changes during the lifetime of an individual? This is a well investigated subject, particularly at the light microscope level [72,82]. Will an SSV analysis be consistent with the prevailing view that indeed it does? And, if so, will an SSV analysis reveal the precise manner in which the structure adapts to prevailing use? There are many other such questions that relate to structural differences within a single bone, and between bones of the same individual, between individuals and between species. We did, for example, note the absence of the bundle super-structure level in rat lamellar bone compared to human bone.

Do all members of the bone family of biological materials have the same number of hierarchical levels of organization? Clearly this is a function of each particular hierarchical scheme, but even within the same scheme the answer is almost certainly that they do not. In the scheme presented in Fig. 3, woven bone, for example, is unlikely to have any higher level of organization beyond level VI. A mineralized tendon appears to be a large-scale unidirectional arrangement of mineralized collagen fibrils, and presuming that it too has bundles, it could be regarded as having only five hierarchical levels. A 3-D study of a mineralized tendon is needed to determine whether or not disordered material is present.

Spiral structures are common in biology [80]. We show here that the coiling motif is reflected at various hierarchical levels in lamellar bone: the triple helical molecules of collagen and the collagen fibrils [83], the fibril arrays within an individual lamella and within the lamellae in a single osteon [70], the canaliculi that have a screw-like conformation in three dimensions [23] and the Haversian canals along the bone [37]. Connecting the lower and higher hierarchical levels are the 1–3 micron diameter bundles that are prominent in the 3-D analyses of human lamellar and fibrolamellar bone [25,26]. In the study of human lamellar bone, a detailed analysis performed on one such bundle raised the intriguing possibility that it has a coiled rope-like structure (Fig. 15) [25]. This, in turn, would imply that the crystal layers inside the bundle are also coiling. In an earlier study, Weiner et al. [45] postulated that the crystal layers rotate from one side of an individual lamella to the other. Are these two observations related? Unfortunately, we are still not able to reconstruct the organization of the crystals in

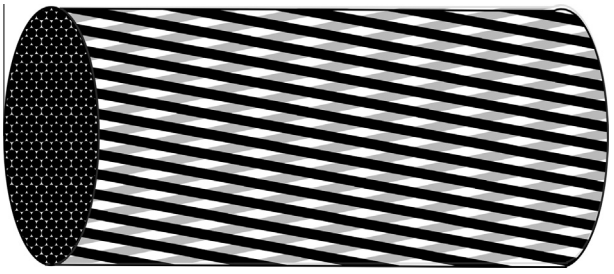

**Fig. 15.** Schematic drawing of the rope-like twisting of the collagen fibrils within a single bundle of unidirectional aligned fibrils.

three dimensions using SSV, as this could provide much insight into crystal organization at different hierarchical levels.

A much debated issue has been whether the crystals in bone are all within the fibrils or between the fibrils, or both [51,52,84,85]. A few studies of different bones using different preparation methods have consistently reproduced images that are interpreted to mean that the crystals aligned with the fibrils are both on the fibril surface and between fibrils [52]. Most studies, however, show or assume that the vast majority of crystals are located within the fibrils. The SSV study and a FIB TEM study of mineralized sections of human bone provide a resolution to this debate: the ordered material has most, if not all, of its crystals located within the fibrils of the close packed fibril aligned arrays, whereas the disordered material is the source of the images interpreted as meaning that the crystals are mainly between fibrils [25]. More detailed studies of the latter are required to better understand the crystal organization in the disordered material.

### 3.2. Bone formation issues

In what sequence are the disordered and ordered materials produced during bone formation? To date there is no direct information on this issue. Following Kerschnitzki et al. [61], it seems reasonable that the cellular processes of the nascent osteocyte first anchor in a preformed substrate, then embed themselves in the disordered material. Finally, the osteoblasts fill in the remaining volume with the ordered material.

It is well established that the osteoblasts produce a preformed extracellular collagenous matrix that subsequently mineralizes. This collagenous matrix could self-assemble in the extracellular space, bearing in mind that it has been demonstrated in vitro that collagen is capable of self-assembly not only into unidirectional arrays, but also into fanning arrays [53]. Alternatively, the fibrils self-assemble inside intracellular vesicles, then the cell extrudes a package of aligned fibrils. An interesting possibility is that the organization of bundles reflects this purported intracellular packaging process. Intracellular collagen containing recesses within the membrane have been observed in forming tendons [55]. Does the observation that bundles are present in human lamellar bone and minipig fibrolamellar bone, but not in rat lamellar bone reflect different modes of self-assembly?

The first formed mineral is deposited inside vesicles within cells that are involved in bone formation [86]. This mineral is highly disordered (usually referred to as amorphous calcium phosphate (ACP)) and in the one case analyzed might be a calcium polyphosphate phase [86]. The mineral-bearing vesicles are extruded into the extracellular collagenous matrix (ordered material) without their membranes, and here the ACP granules appear to disintegrate and very small particles infiltrate into the gaps in the collagen fibril, where they crystallize into plate-shaped crystals [32]. A second mineralization pathway is present in certain bone types, whereby a vesicle is budded off from the host cell, accumulates ACP in the extracellular space and subsequently contributes this mineral to the mineralization process [87,88]. One open question is how the crystals form in the disordered material: is the precursor mineral phase transported in the cell processes and then extruded into the preformed matrix of the disordered material?

The non-collagenous proteins (NCPs) are highly conserved in evolution [89,90], and are hence thought to play key roles in bone formation. The identification of the ordered and disordered materials in bone raises the question of where the different NCPs are located with respect to these two materials, and whether or not they have specific distributions within each of the materials. High-resolution mapping of the NCP locations in three dimensions may soon be possible with the development of cryo-SSV [91], as this will make it possible to label the macromolecule of choice with a gold-labelled antibody and then identify its location in an unstained matrix. In cryo-SSV the density of the matrix is contrasted against the density of the vitrified ice phase in which it is embedded.

### 3.3. Bone function issues related to the presence of ordered and disordered materials

The fact that the cell processes are embedded inside the disordered material makes it likely that it is the mineral of the disordered phase that is directly involved in the mineral homeostasis role of bone. Furthermore, as the osteocyte cell processes are housed in the canaliculi, they will most effectively monitor the strain in their immediate environment, namely in the disordered material. Finally, if the ordered and disordered materials have different elastic and fracture properties, then these need to be understood in terms of the overall mechanical properties of bone the material. We clearly need to learn much more about the structural properties of these two different materials, as well as their mechanical properties, in order to assess their potential significance with regard to the above functions. We also need to determine which bones do indeed have a disordered material component and, in those that do, in which the disordered component is continuous and in which it is discontinuous.

## 4. Concluding comment

A hierarchical structural scheme is a framework for defining basic questions about structure–function relations in bone. The identification of the ordered and disordered materials in lamellar and fibrolamellar bone from several different species has prompted us to redefine existing hierarchical schemes and in this way to better clarify the broader implications of these observations, and to formulate some fundamental questions that arise as a result of this novel view of bone 3-D structure.

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We thank Rotem Almany-Magal for kindly providing the fibrolamellar bone micrograph (Fig. 4).This research was funded by the Israel Science Foundation grant number 407/10. S.W. holds the Dr. Walter and Dr. Trude Borchardt Professorial Chair in Structural Biology.

## Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 7–9 are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.05.024.

## Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2014.05.024.

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