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NANO- AND MICROMECHANICAL PROPERTIES OF HIERARCHICAL BIOLOGICAL MATERIALS

Insights into whole bone and tooth function using optical metrology

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Abstract Understanding the relations between the mechanical responses of whole entities, their materials properties and their structures, is a challenge. This challenge is greatly enhanced when the material itself is complex, and when the entity it forms has a convoluted shape. It is for these reasons that it is still beyond the state-of-the-art to predict and fully understand the mechanical functions of whole biological entities such as bones and teeth. Recent advances in optical metrology open up new opportunities as they enable the precise and accurate mapping of the manner in which the entire surface of a whole stiff mineralized tissue deforms. Furthermore these data can be obtained non-destructively and without contact with the sample. Data of this kind create the exciting possibility of relating the complex distribution of mechanical properties of loaded biological materials such as bone and teeth and their microstructures to deformations and strains. Such studies could improve our understanding of normal physiological processes such as skeletal aging, as well as disease processes such as osteoporosis. They also provide opportunities for engineers designing bio-inspired materials to study the principles, advantages, and characteristics of the behavior of hierarchical and multifunctional materials.

In this manuscript we review optical metrology methods, highlight studies of whole body function for bones and teeth, and in particular those studies that provide insights into structure-function relations. We also outline the potential for future studies.

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Introduction

Understanding the relations between the mechanical responses of whole entities, their materials properties and their structures, is a challenge. This challenge is greatly enhanced when the material itself is complex, and when the entity it forms has a convoluted shape. This situation is almost always the case in biology and was well recognized by D'Arcy Thompson [1] in his classic publication entitled "On Growth and Form." He wrote in 1917 "Matter as such produces nothing, changes nothing, does nothing; and however convenient it may afterwards be to abbreviate our nomenclature and our descriptions, we must carefully realize in the outset that the spermatozooan, the nucleus, the chromosomes or the germ-plasma can never act as matter alone, but only as seats of energy and as centers of force" (p. 20, volume I). Despite the years that have elapsed since 1917, and the enormous technological advances made, it is still beyond the state-of-the-art to predict and fully understand the mechanical functions of whole biological entities such as bones and teeth.

Various attempts have been made to achieve this goal. They were based mainly on measuring deformations of whole bones or teeth at single points, or developing constitutive theoretical models that can predict the behavior of the whole bone or tooth based on the mechanical properties of its individual constituent materials. However both approaches were, for the most part, only partially successful. Recent advances in optical metrology open up new opportunities as they enable the precise and accurate mapping of the manner in which the entire surface of a whole stiff mineralized tissue deforms.

Data of this kind create the exciting possibility of relating the complex distribution of mechanical properties of loaded biological materials such as bones and teeth and their microstructures to deformations and strains. Such studies could improve our understanding of normal physiological processes such as skeletal aging, as well as disease processes such as osteoporosis. They also provide opportunities for engineers designing bio-inspired materials to study the principles, advantages and characteristics of the behavior of hierarchical and multifunctional materials [2, 3].

The materials of bones and teeth

Bones and the bulk of teeth are composed of the same basic materials [4]. Only the thin hard outer layer of teeth (enamel) is quite different [5]. The materials that bones are composed of are also collectively called "bone," whereas in teeth (aside from the enamel) they are collectively called "dentin." They both have the same constituents which include the mineral, carbonated hydroxyapatite (also known as dahllite), the framework protein, type I collagen, many other so-called non-collagenous proteins and water [6]. Enamel also contains carbonated hydroxyapatite, but the crystals are orders of magnitude larger than those in bone and dentin [7]. Furthermore, in most vertebrate teeth (except for those of many fish), collagen is absent in enamel.

The materials of bones and teeth have hierarchical structures, in that they change at different length scales (see Fig. 1). They are also graded materials, as their composition, structure, and mechanical properties may vary continuously or in discrete steps, from one location to another [8]. The combination of these two attributes results in a very complex material type that certainly cannot be described in terms of one value for a particular materials property. The following is a brief description of the hierarchical structures of bone (the material), dentin, and enamel.

The hierarchical structure of bone

"Bone" is a term used to denote both the organ and the material of which it is composed. The material bone is a bio-composite with a complex and hierarchical structure. The various hierarchies of bone span many different length scales. The basic building blocks of the material bone are an organic matrix, most of which is type-I collagen, and a mineral phase (level 1 Fig. 1) [4]. At a higher length scale bone is composed of mineralized collagen fibrils 80–100 nm in diameter (level 2 Fig. 1), arranged in a regular, staggered array of collagen molecules (level 3 Fig. 1) [9]. The crystals are located within and around

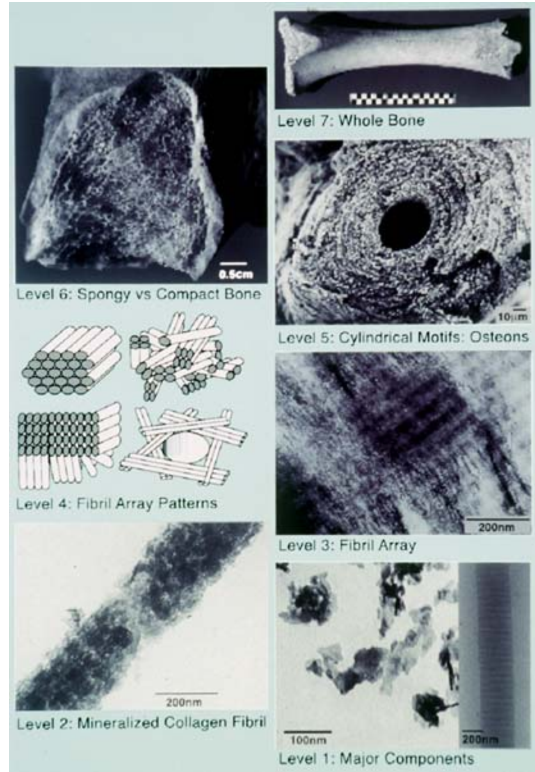

Fig. 1 Hierarchical structure of bone (from Weiner and Wagner [4], with permission)

these fibrils. They are 30–80 nm wide and 2–3 nm thick [10]. At the next hierarchical level the mineralized fibrils are organized in a variety of patterns, the most common of which is the lamella (level 4 bottom left in Figs. 1 and 2). Each lamella is 2–3 microns thick and has a complex rotated plywood structure [11].

At the next hierarchical level, lamellae are arranged in one of several possible ways, depending on location and species; in mature human bone the most common arrangement is concentric layers (called secondary osteons). These form cylinders 150–250 μm in diameter and contain a central hollow tube 80 μm in diameter, which contain blood vessels and nerves (level 5 Fig. 1) [12]. At the next level bone can be mostly solid (cortical) or sponge-like (cancellous) (level 6 Fig. 1). This entire complex arrangement, with different but characteristic 3-D geometric morphologies, ultimately forms the organ bone.

The structures of tooth enamel and dentin

Teeth consist of a crown (the part above the gum line) and one or more roots (the part below the gum line). The crown has an exterior layer of enamel and the root has an exterior layer of cementum. Both parts have a continuous interior layer of dentin, which surrounds the pulp cavity (see Fig. 3).

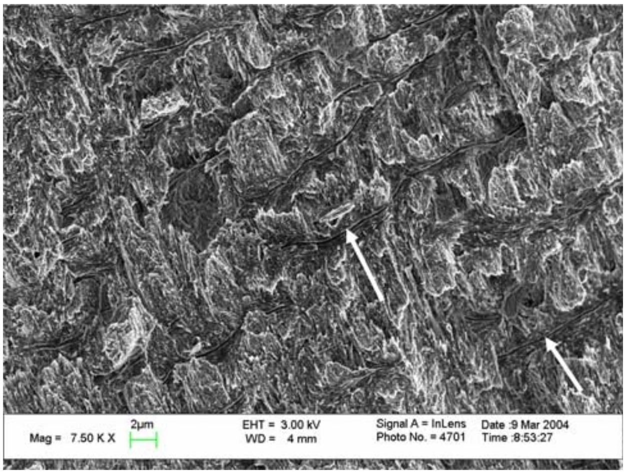

Fig. 2 SEM image of the fracture surface of lamellar bone from a baboon tibia. Different orientations of mineralized collagen fibrils within a lamella can be noted. Canaliculi are shown aligned more or less orthogonally to the lamellae (white arrows)

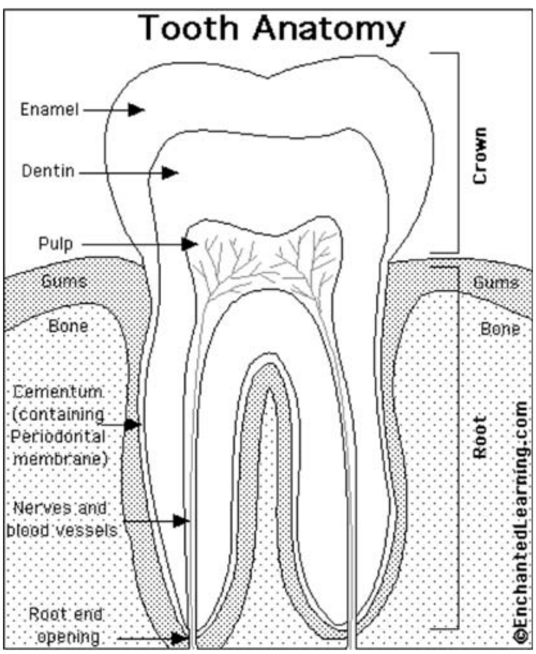

Fig. 3 Tooth anatomy (diagram obtained from: http://www.enchantedlearning.com/Home.html)

Enamel

Enamel consists of 96% mineral, 1% organic material, and 3% water. The basic building blocks of enamel are ribbon-like crystals measuring 60–70 nm in width, 25–30 nm in thickness and their length may span the entire thickness of the enamel layer [13]. They are organized into cylindrical-shaped rods which are surrounded by interrod enamel [5]. Rods are made up of crystals whose long axes are aligned with the long axis of the rod. The interrod region surrounds the rods and in it the crystals are oriented at an angle to the rod. The boundary between the rod and interrod contains organic material called rod sheath [5].

Dentin

Dentin is bone-like in composition, containing by volume around 50% mineral, 30% organic material, and 20% water, making it softer but tougher than enamel [5]. The bulk of the tooth is composed of dentin, which has the same hierarchical structure as bone up to the level of the lamellae. At this level in dentin the mineralized collagen fibrils are all arranged in one plane (more or less parallel to the outer surface of the tooth), but there is little or no preferred orientation within the plane (level 4 bottom right Fig. 1) [14]. This structure is characteristic of the material of the tooth root and part of the crown. In the crown, however, there is an additional dentin type, called peritubular dentin [15]. The peritubular dentin forms a pipe-like lining about one micron thick around the so-called dentinal tubules (Fig. 4a). The tubule diameters are also about one micron. There are huge numbers of such tubules in both the root and the crown of the tooth. The peritubular dentin has little or no collagen and is therefore much denser than the intertubular dentin [16]. At a higher level of organization, the 50 to 200 micron-thick zone beneath the dentin-enamel interface (the so-called dentino-enamel junction or DEJ) has a structure quite different from the other forms of dentin (see Fig. 4b). It is less mineralized than the bulk dentin of the crown and the collagen fibrils have a different organization [17]. As a result, it is less stiff than the bulk of dentin.

The graded structure of bone and tooth materials

The mechanical response of materials with spatial gradients in composition and structure is of considerable interest in disciplines as diverse as tribology, geology, optoelectronics, biomechanics, fracture mechanics, and nanotechnology [8]. The damage and failure resistance of surfaces to normal and sliding contact or impact can be changed substantially through such gradients. The composition, structure, and mechanical properties of a material may vary continuously or in discrete steps with depth beneath a free surface. Gradations in microstructure and/or porosity are commonly seen in biological structures such as bamboo, plant stems, and bone, where the strongest elements are located in regions that experience the highest stresses. Learning from nature, materials scientists increasingly aim

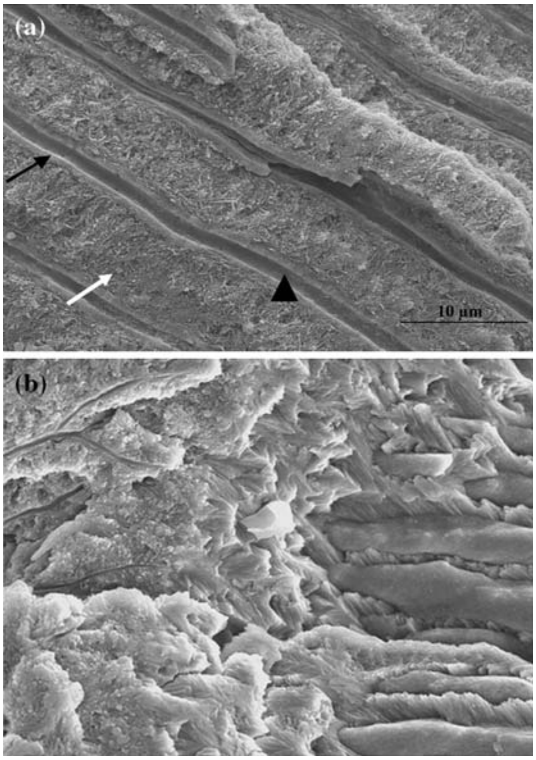

Fig. 4 (**a**) Crown dentin microstructure: Typical scanning electron microscopy (SEM) micrograph of a fracture surface (dehydrated) of crown dentin. The dentinal tubules (black arrowhead) and intertubular dentin (white arrow) can be clearly seen. Crown dentin has an extra phase of highly mineralized peritubular linings surrounding the tubules (black arrow). (**b**) The large enamel crystals can be seen on the right side, and the dentin (including the dentinal tubules) on the left. The DEJ forms the border area between them, in the center of the picture

to engineer graded materials that are more damage-resistant than their conventional homogeneous counterparts [2]. This is particularly important at surfaces or at interfaces between dissimilar materials, where contact failure commonly occurs.

Bone and teeth are both fine examples of naturally occurring graded materials. The graded structure of bone can be seen at several levels. At the osteonal level for example, differences in the level of mineralization occur within osteons, as the inner (younger) lamellae are less mineralized than the outer lamellae. The outer border of an osteon (so-called cement line) is believed to contain very little collagen and is less mineralized than the surrounding bone [12], whereas the so-called interstitial lamellae (remnants of old osteons) are more highly mineralized. This graded structure critically affects the mechanical behavior of bone, and in particular crack propagation, as most cracks tend to advance until reaching cement lines, at which point they stop due to this weak interface and travel around the osteon rather than through it. At another level, the transition between cortical and cancellous bone is also graded, and can be observed grossly in terms of increasing bone porosity from the periosteal to the endosteal surface.

Teeth are also quite obviously graded. Tooth enamel hardness decreases away from the external surface towards the DEJ. At the DEJ hardness drops precipitously for a distance of 200–300 μm (dentinal soft zone), and then partially recovers [18, 19]. As a result, the enamel cap displaces primarily as a rigid body, and the soft dentinal zone serves as a cushion between the stiff enamel and less stiff dentin. Within the crown dentin, the materials properties continuously change from one location to another [20], and the intertubular and peritubular dentin also appears to have a graded interface [18, 21].

Mechanical performance of whole bone and teeth

To date most mechanical studies of bones and teeth have investigated the mechanical properties of each of the bulk materials that make up the bone or tooth. These studies were limited by the minimum size of samples needed for measurements carried out in materials testing machines. As a result osteonal bone is well investigated, as is plexiform bone from bovids. Measurements of other bone types such as circumferential lamellar bone, parallel fibered bone and also root and crown dentin, were infrequently carried out since special circumstances or experimental set-ups are required due to the small size of the samples available. In the last 10 or so years the nanoindenter has been used to measure elastic properties at a very high spatial resolution [22, 23]. This method has greatly increased our understanding of the graded nature of these biological materials and the differences that exist between the mechanical properties of different structural types.

Most investigations of the mechanical performance of whole bones have to date relied on in vivo or in vitro experiments in which bones were tested in compression or by 3- or 4-point bending. Measurements in these experiments consisted of single values like load-to-yield and load-to-failure. Strain measurements were made at a very limited number of points (1–20) by placing strain gauges on the surface of bones and teeth. Alternatively theoretical numerical models (mostly finite element models) were used, which were based on numerous simplifying assumptions, particularly as far as material properties were concerned, and thus yielded rough approximations only. Optical metrology offers an alternative approach which yields data of substantially greater value.

Optical metrology methods

The main advantages of all optical metrology techniques are the full-field nature of the measurements they provide, and that they are non-destructive [24]. In essence a measurement is obtained from each pixel in the field of view. The number of pixels is dependant upon optical resolution, and is usually around 10^5. In this regard, results obtained experimentally by optical methods are similar to results that would be obtained by a very large (and practically impossible) array of strain gauges.

Optical methods provide full-field surface measurements of either stress (photoelasticity) or displacements and strains (various interferometry methods and digital image correlation (DIC)). Full field measurements, be they deformation, strain or stress, have several advantages: they can identify local strain or stress peaks and gradients, which would be missed by measurements made with few strain gauges. This is particularly significant in the case of inhomogeneous, anisotropic, graded biological materials. They are also compatible with the results of finite element analysis, and therefore facilitate model validation. Furthermore, the full-field data set makes it possible to vary finite element models in terms of material properties until the predicted numerical results agree with measured results, providing deeper insight into local material properties.

All optical metrology methods are based in one way or another on interference. Interference is a phenomenon that occurs when two or more waves overlap each other in space. The superposition principle states that the resulting field is the sum of the original fields. The resultant intensity however is not merely the algebraic sum of the intensities but also depends on the phase difference between the different waves. Thus interference may be constructive or destructive.

Here we review optical metrology methods, highlight studies of whole body function for bones and teeth, and in particular those studies that provide insights into structure-function relations. We also outline the potential for future studies.

Photoelasticity

Principle of the method

Photoelasticity was one of the first optical metrology techniques used for the study of whole bones and teeth. The method relies on the property of birefringence that certain so-called photoelastic materials exhibit when subjected to stress. Birefringent materials have two different refractive indices when light passes through them. Furthermore, the magnitude of the refractive indices at each point is directly related to the state of stress at that point. When using this method, the structure under investigation is coated with a photoelastic material, or a replica made of photoelastic material is produced whose geometry is identical to that of the structure investigated. When such a sample is illuminated by polarized light after loading, its birefringence causes the light to become refracted in the two orthogonal principle stress directions. The difference in the refractive indices leads to a relative phase retardation between the two component waves. The two waves are then brought together in a polariscope, and interference takes place. As a result fringe patterns form, which depend on the relative retardation. These fringe patterns allow the determination of the state of stress at the surface of the object tested.

Despite its appeal as a method able to map surface stress distributions, photoelasticity has several inherent weaknesses. When a sample is coated with a photoelastic material, potential errors include a mismatch of Poisson's ratio between the coating material and the investigated structure, incorrect light incidence, uneven coating thickness and the potential reinforcing effect of the photoelastic material [25]. When a replica is used, only the geometry is simulated, and the complex materials properties are not taken into account. Furthermore, replicas or coated models cannot interact in a biologically relevant way with physiologic fluids, thus in essence a dry sample is tested. This further reduces the validity of the experimental results.

Teeth

One of the first applications of 3-D photoelasticity to study the behavior of whole teeth was by Johnson et al. [26]. They measured the stress fields occurring in epoxy resin replicas of human molar teeth with different types of cavity preparations. They showed that when cavities are prepared with rounded angles, stress concentration is minimized compared to cavities prepared with sharp angles.

The nature of the stress distribution from the tooth root to its supporting alveolar bone was investigated by Asundi and Kishen using a combination of in vivo strain gauges and in vitro photoelasticity experiments [27–31]. They used epoxy resin to create a model of a normal tooth and its supporting alveolar bone, which included a thin layer of silicon rubber between the bone and the tooth to simulate the periodontal ligament. Using digital photoelasticity, they showed the stress distribution pattern in the tooth and supporting bone replica (see Fig. 5). The replicas were loaded at several angles relative to the long axis of the tooth. As the loading force direction formed a larger angle with the long axis of the tooth, stresses in the alveolar bone increased. They also showed that stresses decreased from the top (cervical) to the base (apical) region of the root, and that most of the bite forces along the tooth axis caused loading of the alveolar bone in the cervical region, and to a lesser extent in the apical

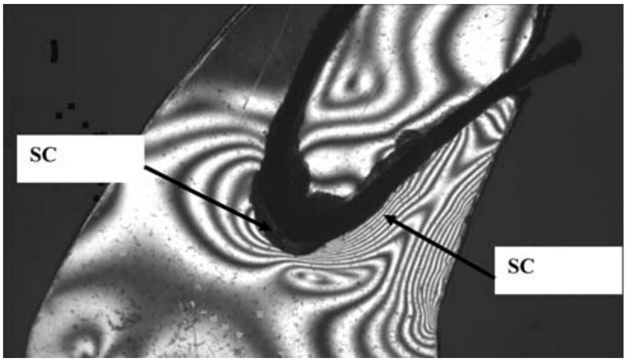

Fig. 5 Stress patterns in tooth and alveolar bone, showing areas of stress concentration (SC—areas of increased density of fringes) (From Asundi and Kishen [27], with permission)

region. They suggested that this might protect the soft tissues (blood vessels and nerves) entering the root at its apex. Thus these studies demonstrate well the use of whole-field results to better understand aspects of the behavior of the entire tooth in its bony socket. This could not be achieved only with strain-gauge measurements.

The above experiments represent the stress distribution based solely on geometry. Kishen et al. [32] overcame this weakness by comparing the distribution of the elastic modulus of dentin (using microindentation on tooth sections) with the stress patterns that developed in the photoelastic model. Dentin was shown to be a graded material, and its properties (elastic modulus, degree of mineralization) varied spatially. The stress pattern observed in the homogeneous photoelastic material predicted bending with compressive stresses on the facial side and tensile stresses on the lingual side. The authors speculated that teeth adapt their structure to the loads they experience, as do bones. However, since teeth do not change their morphology, adaptation occurs in the form of creation of material property variations. These variations allow teeth to avoid areas of high stresses and result in uniform stress distributions.

Kishen and Asundi [33] also used photoelasticity to investigate the behavior under load of individual teeth with and without endodontic treatment. They showed that endodontic treatment resulted in higher tensile stresses and stress concentration in the remaining dentin than stresses occurring under equivalent loads in intact teeth.

These studies elegantly combine photoelastic measurements with other direct materials properties measurements to produce a more integrated understanding of whole tooth behavior.

Bone

Several studies used photoelasticity to investigate bone and bone-implant mechanical behavior [34, 35]. However, since they all used replicas made of photoelastic resin, the effect of bone structure could not be evaluated and results can only show trends, since the material tested is homogeneous and isotropic; a far cry from the complex, anisotropic and graded bone material evaluated.

Murphy and Prendergast [25] demonstrated another potential use of photoelastic-based experiments. They first created a finite element model to compare two methods of attachment of the implant used in shoulder replacement surgery. They then used reflective photoelasticity on 5 scapulae coated with a thin photoelastic layer and loaded as in the model to validate their predictions. The photoelastic and finite element results were similar, creating confidence in the results of their numerical simulation. We note that both the numerical model and the experiment use a simplified representation of the bone (isotropic and homogeneous) hence results must be interpreted with caution and are likely to be different in the in vivo situation.

Deformation-measuring interferometry techniques

Interferometry techniques used for optical metrology are based on illumination of the investigated sample by coherent laser light and on the resulting interference phenomena that occur in various ways. These methods are capable of detecting sub-micron displacements. They include Moiré interferometry (MI), holographic interferometry (HI), and electronic speckle pattern interferometry (ESPI).

Moiré interferometry

Principle of the method

Moiré interferometry requires the application of an adhering grid of parallel lines to the surface of the sample. This grating deforms together with the sample surface. Another identical grating is overlaid on the sample surface. Interference between the un-deformed and deformed gratings in reflected light produces Moiré fringes, from which the two components of displacement at each point on the surface can be determined. Differentiation of the displacement fields makes it possible to also determine the surface strain tensor. Various Moiré methods, such as Moiré fringes and digital MI, have frequently been used to study the strain distribution in bones and teeth under load.

The need to apply gratings to the sample surface of biological materials is the main limitation of this technique, since such application is technically demanding in small samples, may affect the mechanical behavior of the sample, restricts access of water and limits the sensitivity and precision of the resulting measurements. Another limitation is the 2-D nature of this method, since out-of-plane displacements cannot be determined. Higher resolution, non-contact methods such as HI and ESPI overcome these deficiencies (see below).

Teeth

A key insight into the design strategy of teeth was obtained using Moiré fringes on slices of human teeth [18]. The study showed that under compression much of the strain is taken up in a zone some 200 microns thick that separates the stiff outer enamel layer from the crown dentin. This zone was known from hardness measurements to be much softer than the enamel and the underlying dentin. It was proposed that this "soft zone" is an important working part of the teeth in that it acts as a cushion or a gasket during mastication.

Huo [36] extended this study by using a finite element model with the same geometry as the tooth slices. Four finite element models, using different types of material properties for dentin were tested. They ranged from homogeneous and isotropic properties to an inhomogeneous and anisotropic model. The latter model yielded numerical results that most resembled the experimental data produced by Wang and Weiner [18]. Clearly the model can now be used to better understand the structure–mechanical relations of teeth by systematically varying the input parameters and the geometry. This is a very promising use of a finite element model to extend the data and improve our understanding of the functional attributes of this soft zone, or for that matter other design features of a tooth or bone.

A follow-up of the Wang and Weiner experiment was performed by Wood et al. [37] using MI. This experiment was also designed to investigate the material properties of the soft zone, but using a completely different approach. Slices of the tooth crown were cut perpendicular to the long axis of the tooth. In this way the enamel formed a continuous ring around the dentin. In other slices the enamel was not continuous. The changes in shape of the slices were then monitored as a function of changing humidity. The results clearly showed that if the enamel constrained the dentin then very little deformation occurred during drying. If however the enamel was discontinuous, then much of the strain was localized in the soft zone as the dentin dried. This too therefore demonstrated the unique materials properties of this zone. In addition, the experiment showed that a continuous enamel ring prevents the dentin from contracting in this zone during drying. This presumably implies a very strong interface between the dentin and the enamel.

The role of water in the mechanical behavior of dentin was also investigated by Kishen and Asundi [38]. Several human incisors were ground to prepare parallel-sided longitudinal sections, and a high-frequency grating was attached to the specimen surface by a thin layer of epoxy. The dentin was divided into an outer region (close to the enamel) and an inner region. They showed that when hydrated, both regions underwent the same lateral strains (along a line perpendicular to the long axis of the tooth). However, when dehydrated there was a significant difference in lateral strain between the two regions (higher in the outer region). They found that dehydration of dentin induced residual compressive strains in the outer region of dentin, as was observed by Wood et al. [37] for unconstrained dentin. This showed that free water has a significant impact on the stress-strain response of dentin in a direction parallel to the tubules and perpendicular to them. The presence of water increased the toughness of dentin, while loss of water increased its stiffness. The authors point out that the dentinal collagen fibrils are made of microfibrils separated by spaces filled with water molecules. Dehydration of the dentin leads to loss of these spaces and allows the collagen polypeptide chains to contact each other, and form molecular associations not formed in the hydrated state. These associations stabilize the structure and increase its stiffness.

Moiré interferometry has also been used to determine the deformation gradients of enamel and dentin [39]. The purpose of this study was to look for a biomechanical basis for a significant problem affecting the teeth of humans, namely non-carious cervical lesions, by studying the strain distributions in enamel and dentin in human teeth loaded in compression. They observed that while the enamel underwent marked strain gradients in the direction perpendicular to the long axis of the tooth, the crown dentin experienced marked strain gradients along the tooth axis (see Fig. 6). As load increased, the strains in the enamel increased in the area above the base of the crown (cemento-enamel junction), while strains in the dentin increased below the cemento-enamel junction. These unique in-plane strain patterns led the authors to hypothesize that loading caused by biting forces contributes to strain concentration at the junction between the crown and the root of the tooth. This in turn could cause the formation of cavity-like lesions in this area which are attributed to mechanics rather than bacterial activity.

This example illustrates the ability of optical metrology methods in investigations that aim to gain a fundamental understanding of the design strategies of mineralized organs like teeth and bone. In particular the mechanical behavior that results from the complex interplay of the organ's external morphology and internal structure, combined with the spatial distribution of material properties can be measured. Such data helps explain the normal behavior of teeth, and the occurrence of common lesions such as non-carious defects in the enamel at the crown-root junction.

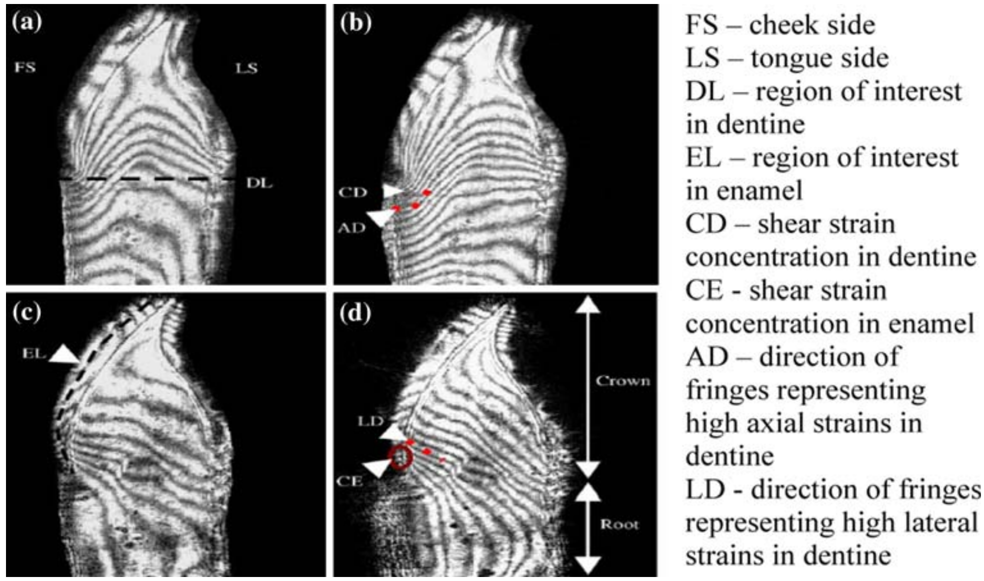

Fig. 6 Typical digital MI fringe patterns in directions along (**A**, **B**) and across (**C**, **D**) the long axis of the tooth, for loads of 10 N (**a**, **c**) and 30 N (**b**, **d**) (from Kishen et al. [39], with permission)

Holographic interferometry

Principle of the method

Holography is a general term describing techniques for recording and reconstructing wavefronts. Holograms are patterns produced by the interference of object and reference beams and recorded on a recording medium [40]. Holographic interferometry allows the comparison of wavefronts recorded at different instants in time. When HI is used to measure the surface deformation of loaded samples, a hologram of the unloaded sample is obtained and stored. It is then allowed to interfere with a wave scattered from the loaded, and therefore deformed sample. The phase difference between the pre-load and post-load wave fronts is calculated, and this describes the deformation that occurred during this time interval.

Early applications of this technique used photographic plates as the recording medium. This process was time consuming, especially the development of the photographic films required. In recent years CCD sensors and powerful computing resources made it possible to directly record the holograms and evaluate them digitally by computer simulation (digital HI). Two procedures can be used: (1) Double exposure interferometry, in which two exposures of the sample are made on the same hologram, i.e. pre- and post-loading. By reconstructing the hologram, the two waves scattered from the sample in the 2 states will interfere. (2) A single recording of the sample in the reference state is made, and then the hologram is processed and replaced in the same position as in the recording. By looking through the hologram it is now possible to observe the interference between the non-constructed sample and the wave from the sample in its original position. Thus deformation can be followed in real time by observing dynamic changes in the interference pattern.

Digital HI is very sensitive to vibrations, and considerable amount of noise may be present in the data. This is especially true when relatively low-resolution recording devices (CCD camera or complementary metal-oxide semiconductor (CMOS) sensors) are used. When samples are measured under water currents and vibrations in the water increase the difficulty of obtaining reliable results. Noise reduction can be achieved by using faster, higher resolution recording devices, and taking all precautions possible to damp out vibrations.

Bone

One of the first reports of the use of double exposure HI described the measurement of strains on the surface of artificial femora made of glass fiber epoxy composite. The femora, with and without implants, were loaded in compression along their long axes [41]. The authors showed that implantation resulted in significant changes in the femoral strain patterns. In particular, significant decreases in peak strains in the upper half of the bone (the region which contains the implant) were observed. This observation confirmed the suspected phenomenon of stress shielding in implanted bones, where the implant, which is much stiffer that the surrounding bone, bears most of the stress, and the bone experiences lower than physiological stress. This causes bone loss (the biologic response to lower load) and makes the bone more susceptible to fracture under non-physiologic impact loading, like a fall. It should be noted that the behavior of femoral replicas made of homogeneous and isotropic material, only yields information on the effect of the bone's geometry on the strain fields.

Double-exposure holography was also used to determine bone deformation (swelling and shrinking) due to thermal stress [42]. Thermal stresses were used simply because they were simple to apply. The experimental results were compared to measurements made with a thermo-graphic measuring system, and a good correlation was found between the temperature and displacement distributions. It was also shown that the deformation fields differed depending on which point was used for the application of the heat source.

Osten and coworkers [43, 44] reviewed the potential of HI in biomechanics. They described a system, which uses two laser sources and two fast CCD cameras. The latter concurrently record the out-of-plane and in-plane deformation components. The deformation fields of notched samples of antler bone were followed dynamically at a rate of 30 hologram pairs per second. The deformation maps clearly demonstrated the presence of high displacement gradients (equivalent to strains) at the tip of the notch (See Fig. 7). Further refinement of this system, using ultra-fast and high-resolution cameras and use of three lasers and three fast cameras will allow experiments to follow fracture phenomena in hitherto unattainable detail and obtain all 3 displacement components, thus significantly extending the potential application of HI to the study of biologic samples.

Electronic speckle pattern interferometry

Principle of the method

Another optical method which allows whole-field measurement of displacements of optically rough surfaces is electronic speckle pattern interferometry. It is based on the observation that when a rough surface is illuminated by a coherent source of light, the reflected light beams interfere locally with each other, creating a grainy image termed a speckle pattern. The speckle pattern changes when the specimen is loaded. The differences between patterns of images obtained before and after applying load are used to detect shifts in the phases of speckles [45, 46]. These phase differences correspond to surface displacements, and their magnitudes can be determined using one of several available phase-shifting algorithms. Phase unwrapping allows the determination of the entire displacement field. One of the main advantages of this method is its extreme sensitivity, which allows detection of displacements as small as tens of nanometers. (The limit of detection is ~λ/30, were λ is the wavelength of the laser light; typically several hundreds of nanometers). There are several additional ways to utilize speckle-based methods. One such method, called shearography, deserves special mention, because it allows direct measurements of displacement derivatives, which can be related to strains. This method is based on causing interference between rays returned from two neighboring surface points. When two such points lie on the same Cartesian axis and are very close to each other, the relative phase change occurring before and after deformation can be related to the partial derivative of the deformation along that axis. One of the advantages of this setup is its reduced sensitivity to vibrations [47].

The potential of ESPI to measure deformations of loaded biomineralized tissues such as teeth and bones increased significantly after it was demonstrated that such measurements can be made with the sample under water [19, 48, 49]. This is essential for the investigation of biological specimens. This technique allows not only the investigation of deformations and strains of entire organs, but also the determination of the elastic properties of millimeter-sized samples with good precision and accuracy in teeth [48] and bone [49]. Furthermore, by improving the signal-to-noise ratio it will be possible to determine local strain variations within these small samples and correlate them with microstructures.

Electronic speckle pattern interferometry has several limitations. It is extremely sensitive to ambient vibrations, and requires an isolated environment, which usually includes an acoustically insulated system mounted on top of a floating optical table. Applied loads must be small to avoid large displacements which could lead to decorrelation. Therefore when larger loads are of interest, they must be reached by a series of small and consecutive incremental loads, with displacement fields determined for each increment, and then summed.

Another technical difficulty is the fact that fluctuations in speckle intensities may occur during the time between obtaining the reference image and loaded image, especially when wet biologic samples are tested, resulting in decorrelation. This makes data analysis very challenging. Kirkpatrick and Brooks [50], in an attempt to overcome the difficulties associated with speckle fluctuations, used a modification of classical laser speckle data analysis, which was based on a sequential sampling algorithm which is insensitive to slow speckle decorrelation (relative to the sampling rate). In a preliminary proof-of-concept type of experiment, they showed that when a sample interval of 0.02 s was used, reliable results could be obtained. They applied this technique to obtain the Young's moduli of samples of porcine cortical bone. Another approach was based upon applying very small incremental loads between successive images (reference and loaded state), thus creating displacements which are much smaller than the pixel dimensions. In this technique each experiment consists of many individual steps, and avoids decorrelation. Using this approach, Young's modulus (human dentin and equine cortical bone), and Poisson's ratio (equine cortical bone) were measured [48, 49].

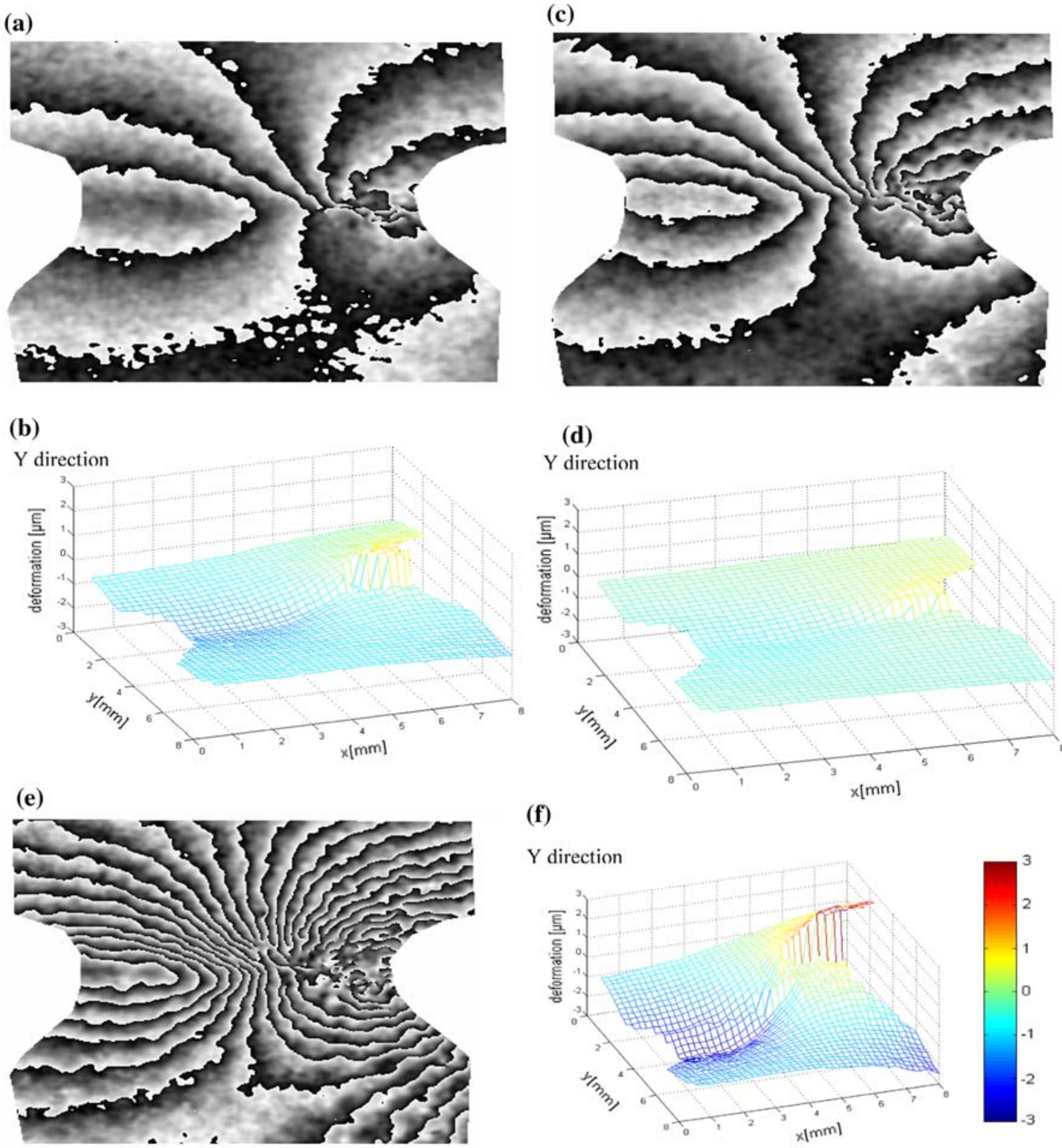

Fig. 7 Deformation of a notched piece of antler bone immersed in water, measured by digital HI, at three different times (0.5, 1, and 1.5 s after the beginning of the loading process). (**a**), (**c**), and (**e**) are the phase maps and (**b**), (**d**), and (**f**) are the corresponding deformations in the *y*-direction

Last, obtaining the three components of displacement requires changing the laser configuration during measurements, and this process requires time. This makes ESPI less suitable for dynamic measurements. A novel approach taken to shorten the acquisition time of in-plane and out-of-plane deformations was reported by Rodriguez et al. [51]. They developed an ESPI system which allowed easy, precise and rapid alteration of the direction of the laser beams illuminating the surface of the object, by use of optical fibers attached to a rotating platform to split and guide the light beam. To demonstrate the abilities of their system, they measured the surface displacements of a human lower jaw bone (mandible) resulting from two different loading locations. However in this preliminary study only the general low-resolution pattern was evaluated and quantitative displacement data were not obtained. It should also be noted that this set of experiments was performed on dry specimens, and therefore does not represent the real deformations this bone will undergo under physiologic load.

Teeth

Zaslansky et al. [19] used ESPI to measure the deformation distributions of whole human premolar teeth (see Fig. 8).

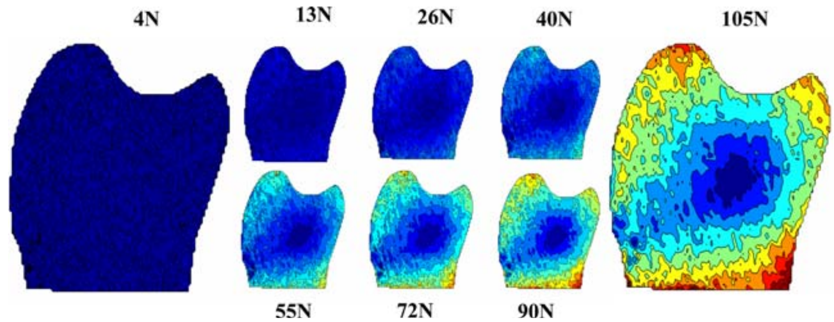

Fig. 8 RDM maps showing deformation of a premolar during incremental loading (From Zaslansky et al. [19], with permission)

Experiments were performed in a custom-designed micromechanical compression device. The entire setup was located within a sealed stainless-steel chamber, and the teeth were submerged in water during the experiment (see setup details in [48]).

Compressive load was applied incrementally either to a localized area at the tip of the main cusp (simulating hard food mastication) or over most of the upper region of the main cusp (simulating soft food mastication). The same protocol was also applied to exact replicas of the tested teeth. It was hypothesized that any difference in the deformation patterns between the real tooth and its replica will be the result of the tooth structure. In order to interpret the spatial distribution of the whole-field displacements a novel approach was developed, named relative displacement maps (RDM). These provide a reference-independent map of the 3-D surface displacements (see [19] for details).

The teeth and their replicas deformed in roughly the same manner. This indicates that the overall deformation pattern of the premolar is mostly controlled by shape, and to a much lesser extent by its complex internal structure. An interesting difference was seen between the deformations occurring due to soft food and hard food mastication. When subjected to a more concentrated load (simulation of hard food) the tooth undergoes substantial bending. Another significant finding was the much more uniform deformation of natural teeth compared to their replicas. This was attributed to the properties of the enamel cap that behaved mainly as a rigid body, with only moderate deformation at higher loads. One particularly interesting observation was that the location of minimal deformation of the natural teeth, but not the replicas, was in the region of contact between neighboring teeth. This is presumably a "built-in" design feature to minimize abrasion between teeth.

Bone

Electronic speckle pattern interferometry has been used to study the three-dimensional strains on the surface of mice femora when load was applied either along the long axis of the femur by compressing the femoral head, or from medial to lateral surfaces at the knee region [52, 53]. It has shown that the two different modes of loading resulted in very different patterns of strain distribution on the surface of the entire femur. In these studies the femora were tested dry, and therefore the results may not represent their true physiologic behavior. Studies such as this further understanding of the response of whole bone to load. They also provide insight into the remodeling process that occurs when a change in loading pattern is applied to bones.

Another potential use of ESPI was recently reported by Mohr et al. [54]. They assessed the process of fracture healing by harvesting mid-sagittal sections of fracture callus (a layer of cartilage and bone produced by the body to bridge the fracture area) from sheep 8 weeks after fractures were created in the middle of their sheen bone (tibia). The fractures were stabilized with an external metal device (external fixator frames). The slices were subjected to axial compression, and the strain distribution in the entire callus was measured by ESPI. Slices with complete bony bridging were shown to have higher stiffness than those consisting mostly of connective tissue bridging. Highest strains in all sections were seen in the fracture gap. Continuation of this study could significantly further understanding of the fracture healing process and provide answers to such questions as the mechanical consequences of the sequence of events occurring during the fracture healing process, and the most suitable conditions required for successful healing.

Digital image correlation

Principles of the method

Digital image correlation is an optical metrology technique which is based on entirely different principles from those of interferometry-based techniques described thus far. Digital image correlation is performed by matching high-resolution structural patterns or color-sprayed random image patterns of the surface of objects before and after they are placed under load. Displacement fields can be determined by using photogrammetry to compare images of the sample before and after load application. An array of measurement points is digitally superimposed on the image of the sample in the pre-load state, to define the locations of displacement measurement. The image processing system stores a small subarea based on its unique surface texture surrounding each measurement point in the preload image (either the inherent texture of the sample or by spraying a contrast agent), and a search is performed in a defined area in the post-load, deformed image. The displacement search procedure is performed by one of a variety of image correlation algorithms. The algorithm determines the position of the deformed points with respect to the undeformed image. The method is not sensitive to rotations; however it detects rigid body motion and deformation. The resulting deformation fields can be smoothed and differentiated, thus yielding the surface components of the Lagrangian strain tensor.

Digital image correlation has several advantages over measurements by ESPI. It is much less sensitive to ambient vibrations, can detect rigid body motion, can simultaneously measure 3-D displacements and has a high dynamic range (microns to millimeters). Its main disadvantage compared to ESPI is its lower sensitivity, which is determined by the field of view and does not exceed 0.3 microns [47, 55].

In the last decade several research groups reported on a wide array of applications of DIC for the measurement of displacements and strains of loaded biological tissues. The most-often investigated tissues were cortical bone, cancellous bone and articular cartilage; other tissues were also studied, from individual cells to large tissues such as the wall of bovine arteries, the bovine hoof horn and the bone-cement-implant system [56].

Bone

The group of Nicolella and co-workers published a series of elegant papers, in which they examined the high-resolution strain distribution within small samples of cortical bone [57–60]. In these studies they have associated and related local strain variations to meso-scale structural elements such as osteocyte lacunae and microcrack tips, and compared them to the global strain of the entire sample. They found that for global sample strains at ~2,000 microstrain, the local strain at the osteocyte lacunae reached values as high as 12,000–15,000 microstrain, and even ~30,000 microstrain near crack tips (see Fig. 9). These studies demonstrated that while the global strain, as measured by strain gauges, is in the physiologically accepted range (2,000 microstrain), much higher strains are measured locally. This observation has a major impact on the various strain-driven theories of the remodeling process.

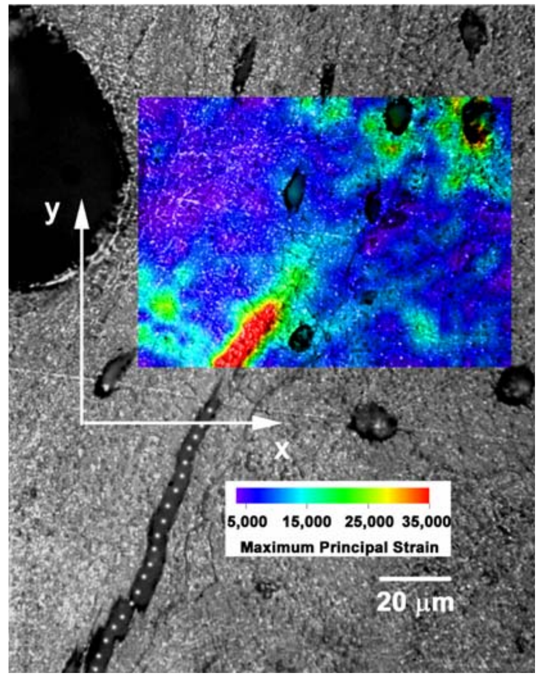

Fig. 9 Local strain distribution overlaid on a photomicrograph of cortical bone. *Note*: Principal strain peaks near osteocyte lacunae and microcrack tip (Figure kindly provided by Prof. Nicolella)

To date, DIC has not been used to measure deformations of entire bones or teeth. However the advantages of this method over interferometry methods (especially the low sensitivity to vibrations and ability to measure large deformations) make it particularly suitable for this purpose.

Discussion

The study of the mechanical behavior of whole organs (bones and teeth) is only at its initial stages. While it seems reasonable to assume that the extremely convolute three-dimensional geometry and intricate material distribution and architecture of bones and teeth, and the hierarchical, complex and graded nature of the materials of which they are made, all play important roles in their response to mechanical loads, real understanding of these relationships is currently lacking. In the past studies of such questions were hampered by the inherent limitations of the available methods of investigation.

The application of advanced optical metrology techniques to the study of biomineralized tissues has therefore much future potential. The addition of such recent technological advances such as extremely fast CCD cameras which can obtain images at rates of up to 10 KHz, and readily available powerful computing power which can easily handle vast quantities of data in real time, will allow researchers in the field of whole-bone and whole tooth function to use interferometry techniques and DIC to answer questions which seemed beyond our reach only a short time ago.

The most basic and intriguing question is that of the deformation (and resulting strain) distributions of whole organs under various types of load. Until recently it was considered impossible to elucidate the strains and stresses occurring in bones and teeth of complicated shape and structure. As has been shown in the preceding sections, while optical metrology techniques show great promise in this respect, attempts to use these methodologies to investigate this question are only beginning.

Bones come in an impressively large assortment of shapes and sizes. Within each bone the different hierarchies are themselves distributed in a very varied way. There are areas which are more mineralized than others, areas with secondary osteonal bone while other areas have primarily parallel lamellae, areas with thick cortex and others with very thin cortex, areas with cancellous bone or without cancellous bone, and so on. It is reasonable to assume that most of these variations can be rationalized in mechanical terms, but until now such rationalizations could only be based on speculation and very little experimental support. Testing whole bones with optical metrology methods can allow a much more detailed exploration of the mechanical significance of the shape and structure of bones.

The organ bone must be both *stiff* (to resist deformation under the action of muscles) and *strong* (to bear load without breaking). A further requirement is the basic need to minimize weight so as to decrease the energy cost of locomotion. The solution nature found to these conflicting demands is a structural motif common to almost all bones in all species—an outer cortical shell of varying thickness, which surrounds cancellous bone in some regions. Cortical bone is almost solid, with very few voids in it, while cancellous bone (also termed spongy or trabecular bone) consists of slender (50–150 μm in diameter) and relatively long (1–2 mm) struts surrounded by large spaces. It has been shown that cortical and cancellous bones are made of the same *material*, possibly with minor differences in the level of mineralization. However due to the different void contents, a volume of cortical bone is much stiffer (10–100 times) than an equal volume of cancellous bone. Therefore it is commonly believed that most of the stiffness of bones is due to their cortical shells. The cortico-cancellous structural arrangement raises the question: *what is the mechanical function of the cancellous bone component?*

Only a small number of studies tried to address this question experimentally, and results were limited due to methodology limitations. Using optical metrology it is now possible to obtain a quantitatively precise and accurate answer to this question. Bones can be scanned to determine with high resolution their structure (in terms of voids, mineral content, sizes, and orientation of cancellous struts, etc.), and then loaded while an optical measurement method is used to measure surface displacements. Since the methods are non-destructive, the load can be removed and the bone treated to remove a predetermined amount of cancellous bone from a selected region (quantified by a post-removal microCT scan). The loading experiment can now be repeated, and surface displacements of the modified bone measured. Direct comparison can be made between the pre- and post-removal displacement and strain fields, showing quantitatively the effect of the removal of cancellous bone regions. Preliminary results of such an experiment demonstrate an obvious difference in strain distributions between the two states in a rat femur.

One of the most outstanding attributes of all optical metrology methods is their compatibility with the results obtained by finite element analysis. The finite element method (FEM) is a commonly used engineering numerical method which is used to determine the stresses, strains and deformations of structures of complex geometry and material properties when placed under load. Parametric analysis of the contribution of each component can be easily achieved by this technique. FEM is frequently used to model various biomineralized tissues, such as bones and teeth [36, 61–65]. In such models, the geometry, even when complicated, can be simulated very accurately by advanced CAD tools. However the need to assign correct material properties to the elements of these models poses a much greater challenge since biologic materials are anisotropic, inhomogeneous and graded in nature. Thus it is possible to create a model of a bone or tooth, and then experimentally test the *same* bone under conditions identical to those simulated in the numerical analysis, measuring displacements with optical metrology. It is then possible to compare the analysis results to the experimental results. The model can be modified and fine-tuned up to the point when the predicted and experimental results are close, indicating by reverse engineering the correct spatial distribution of the material properties within the bone or tooth. Several simple examples of this approach were provided above ([25, 36]), but the potential is far from tapped.

Optical whole-field metrology methods can also be applied to good advantage in studying questions arising in clinical settings. For example, dentists are not certain of the effect carries, or various restoration materials and methods used to treat them, have on the mechanical behavior of whole teeth. To answer such questions, we are currently testing intact premolar teeth under load with ESPI. We first measure the deformation maps occurring as a result of load applied to intact teeth. We then create carries-like lesions in their crowns and retest them. We then use standard filling materials and techniques and test them a third time (Barak et al., submitted). It should be stressed that such an endeavor requires a system which is able to accurately and *non-destructively* measure whole-field displacements of objects immersed in water. ESPI and other optical metrology methods are therefore ideally suited to achieve these goals.

Other biologically intriguing questions involve load transfer between bones through joints and between teeth and their sockets through the periodontal ligament. Optic metrology methods could supply detailed answers to questions of both basic and clinical nature. Such investigations are particularly difficult because of the strongly viscoelastic nature of the soft tissues involved in load transfer.

An area which has received much attention in recent years is the phenomenon of microcrack propagation in bone, up to fracture, and in particular the as yet unresolved question of how cortical bone is able to withstand fracture, either after long-term cyclic loading, or during trauma. The answer to this question has far-reaching implications to human health, since failure of protective mechanisms is relatively common, especially in young active athletes and in the aging population. It has become increasingly clear that the fracture process in bone will have to be modeled using non-linear fracture mechanics models, rather than linear elastic ones, because in bone the size of the process zone is very large compared with the size of the crack. However, direct experimental evidence for these models is lacking. This need is particularly suited to the use of ultra-fast cameras and digital HI which will enable dynamic measurement of the deformation and strain fields associated with crack propagation near the tip of the crack, in an area in which linear elastic predictions fail.

Conclusions

Optical metrology methods make it possible to study and obtain data on the mechanical behavior of entire structures such as whole bones and whole teeth. These data combined with numerical modeling could contribute significantly to our understanding of the challenging questions concerning structure-function relations in these important organs.

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