Multiscale visualisation of tooth-periodontal ligament-bone fibrous joint function

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Several experimental methods are being used to investigate biomechanics of joints and their associated tissues. This article examines how *in situ* loading coupled with X-ray microscopy enables visualisation of internal structures of intact joints (mineralised tissues interfacing ligaments) under physiological loading conditions. X-ray imaging of the tooth-periodontal ligament (PDL)-bone fibrous joint, complemented with electron and light microscopy techniques, were used to provide insights into (1) the "functional osseointegration" aspect, should the tooth be replaced with an implant to regain chewing function, and (2) the role of softer vascular components within the ligament toward the regenerative potential of the PDL.

Introduction

Load-bearing joints in most organisms are constructed with hard and soft tissues. From an engineering perspective, hard and soft tissues act as structural components, absorb loads, and enable motion. Spaces in between structural components provide degrees of freedom for joint movement and consequently enable motion of an organism. In order to investigate the functional biomechanics of a joint, the strength related characteristics of its structural components are often investigated at several hierarchical length scales [1-7]. Mechanical tests are also performed on specimens which have been reduced to standard dimensions, providing useful material behavior data, [8-12] but with limited contextual information.

Throughout this article, we will provide a holistic approach to address the objective of developing a link between joint biomechanics and observed deformations within tissues, as well as the site-specific expression of molecular constituents by cells. This will be demonstrated using an *in situ* loading device coupled to an X-ray microscope [13]. *In situ* loading through visualisation provides invaluable insights into biological processes within load-bearing joints subjected to day-to-day functional activities (e.g. walking, chewing) or aberrant loads (e.g. clinical interventions) [14, 15].

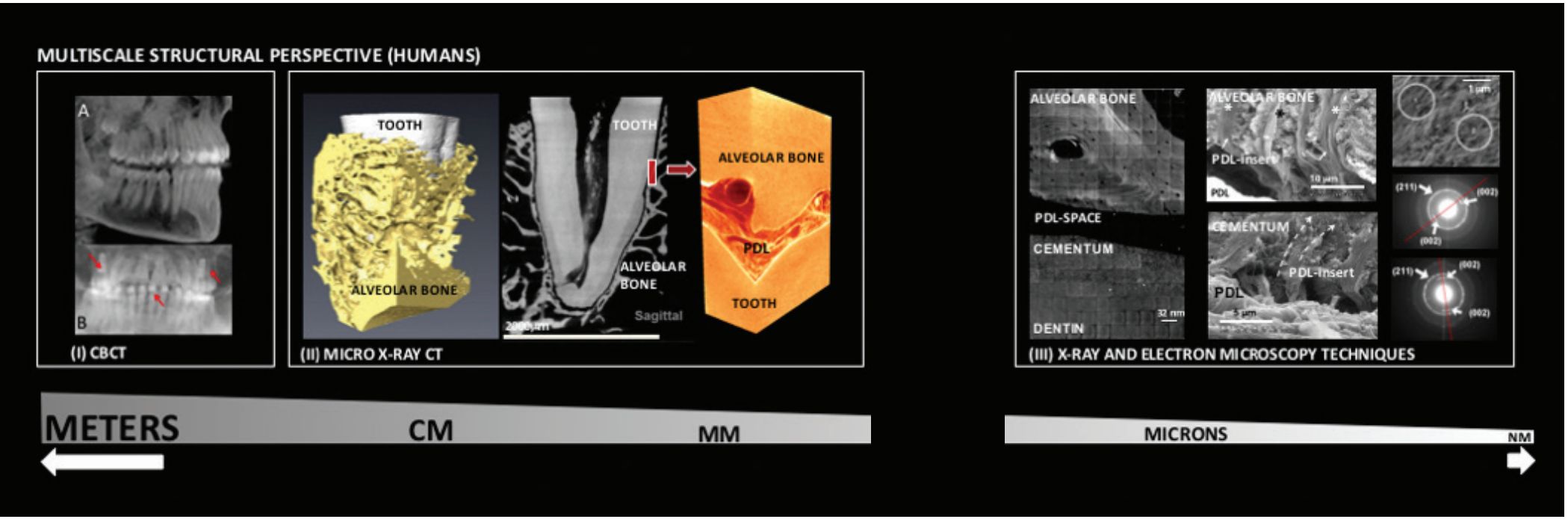

Figure 1. *Multiscale imaging of the tooth-PDL-bone fibrous joint [left to right]: (I) Patient care X-ray cone beam computed tomography illustrates the jaw, location of teeth, and the plane along which maxillary teeth interdigitate with mandibular teeth. Red rectangle in I illustrates a premolar that is imaged at higher resolution using an X-ray computed tomography unit (II). (II) X-ray microscopy with the use of a contrast agent revealed blood vessel channels within the ligament. Black rectangle in II illustrates the tooth-PDL-bone complex (III). (III) High resolution X-ray microscopy revealed heterogeneity in structure and mineral density within bone, cementum and dentin. Scanning electron microscopy illustrated organic ligament-inserts (asterisks) into bone and cementum.Transmission electron microscopy along with selected area electron diffraction revealed ultrastructural information of the mineral (crystal) and its structure relative to the organic fibrils. CBCT- cone beam computed tomography; CT – computed tomography; PDL-periodontal ligament*

Stressing tooth and bone

In this article, the organ of interest is the dental complex, where a seemingly monolithic tooth is attached to an alveolar bone with a fibrous periodontal ligament (PDL), collectively known as a fibrous joint or gomphosis [16]. Visualisation and contextual analysis of hard (tooth and bone) and soft (PDL) structural components under function is enabled by *in situ* X-ray microscopy [13]. Insights into the adaptive nature of the radiopaque cementum and alveolar bone, and the radiotransparent PDL are highlighted to illustrate their plausible regenerative capacities. These insights open avenues for regenerative medicine specific to a tooth-PDL-bone complex where impairment can occur due to periodontal disease [17] or aberrant forces. The bulk of our studies use *in situ* X-ray microscopy to visualise internal structural components under tension or compression using a specially designed mechanical stage housed within an X-ray microscope.

We illustrate here the importance of *in situ* imaging through two cases: (1) implant-bone contact that provides insights for the development of effective methodologies for implant-bone integration, where the tooth is substituted with an implant to regain chewing otherwise lost to disease [18], and (2) the role of the ligament between the tooth and bone, and the regenerative potential of the complex [19-21].

Periodontal ligament

The tooth is attached to the alveolar bone through a vascularised and innervated periodontal ligament (PDL). From a biomechanical standpoint, the softer, water-retaining ligament acts like a cushion, absorbing and dampening chewing forces placed on the biting or chewing surface of a hard tooth. From a physiological standpoint, the ligament delivers nutrients to the ligament-bone and ligament-cementum attachment sites (Figure 1). Nutrients preserve fertile landscapes at the attachment sites and stimulate cells to maintain a balance between matrix molecules facilitating mineral resorption and formation events [19, 22]. The delicate balance between formation and resorption of minerals in turn promotes an adequate joint space necessary to absorb chewing loads. Much like a closed loop control system, the nerves in the ligament provide feedback for load regulation that would otherwise cause tooth/bone fracture. In addition to providing nutrients, the vascular components within the ligament also serve their potential role as reservoirs for progenitor cells, highlighting the regenerative capacity of the ligament and the periodontal complex. Despite the richness of the PDL alluded by its regenerative potential, limited research has been done on relating the effect of mechanical forces to its regenerative capacity and thereby maintenance of the joint in the chewing process.

Implants

Chewing capability is often restored using titanium-

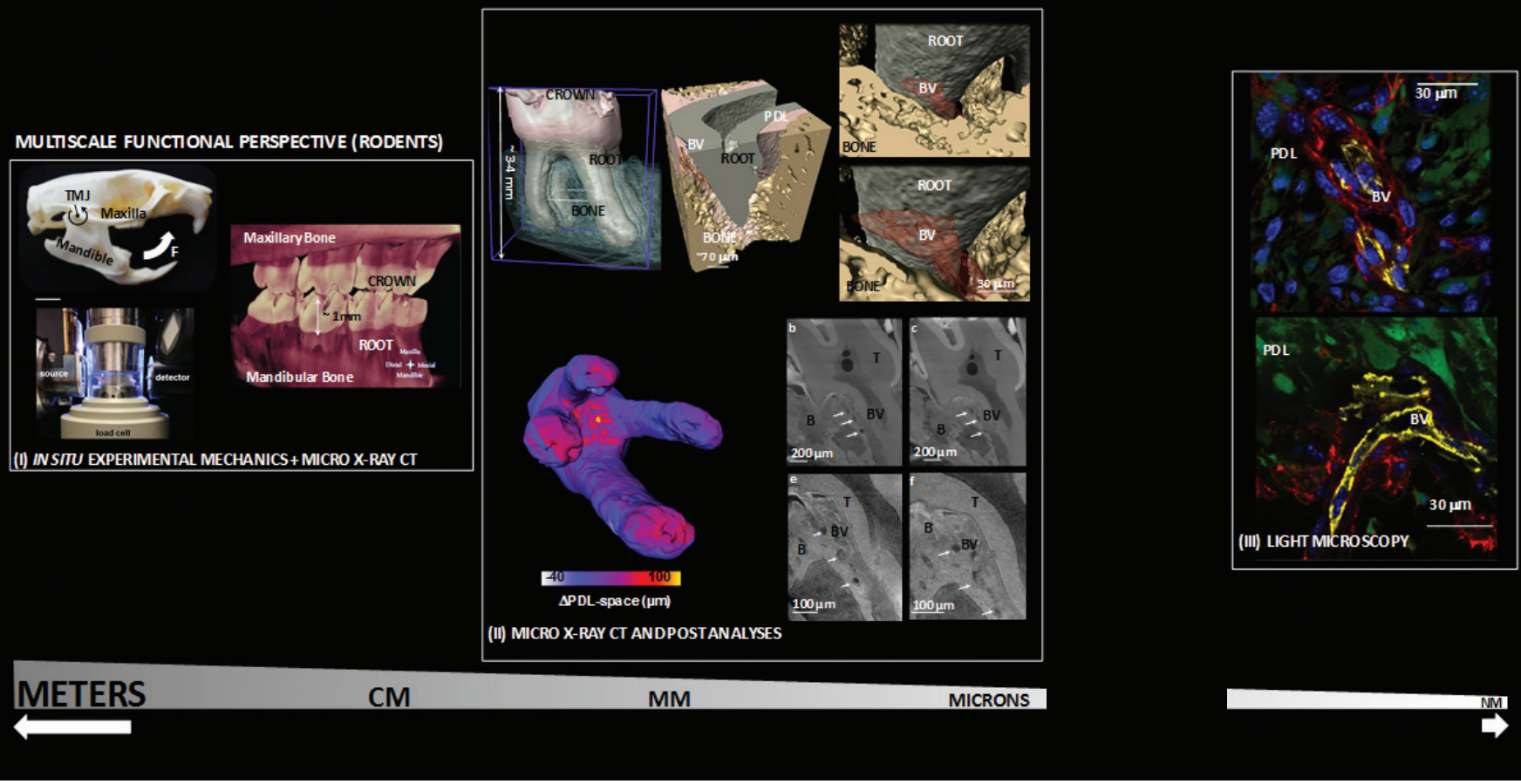

Figure 2. *Clinically observed conditions are mimicked in small-scale animals to investigate changes in tissues and in joints as a function of length and time [left to right]: In situ loading of an intact tooth-ligament-bone complex (I) illustrates narrowing and widening of the ligament-space mapped on the root surface (PDL-space), and deformation of blood vessel (BV) within the ligament (II, arrows). A larger BV is digitally isolated and is shown between the root and bone. (III) Under load, the blood vessels within the organic PDL experience mechanical strains and its effect in differentiation of stem cells is further interrogated through high resolution light microscopy of fluorescently labelled tissues (blue: cell nucleus; yellow: CD31+ indicating endothelial cell localization; red: NG2+ indicating progenitors; green – Scleraxis expression in fibroblasts of the PDL). PDL-periodontal ligament; BV – blood vessel; TMJ – temporomandibular joint; F – gnawing or biting forces on the incisors of rodents.*

based implants. Integration of the implant with alveolar bone is vital to restoring function. Implant-bone integration continues to be interrogated at various hierarchical length-scales (macrometer through nanometer), but with limited information derived within the context of function. Through the use of an experimental mechanics technique, we interrogate osseointegration [18]. To better understand regeneration, implant-bone integration, and eccentric loading on a tooth, we employ an *in situ* loading device coupled to a high resolution X-ray microscope. The hybrid method of combining biomechanical testing with X-ray imaging enables visualisation of internal structural components under simulated physiological conditions. With the use of mechanical loads applied *in situ*, the functional relevance of visualised structures can be established.

Materials and Methods

Specimen procurement and preparation of human and rat specimens were done as per the guidelines by the Committee on Human Research (CHR) and the Institutional Animal Care and Use Committee (IACUC) at UCSF. Freshly harvested specimens were prepared for *in situ* mechanical testing as described previously [14, 15, 18]. Biomechanical testing was done on human specimens obtained through the Willed Body Program at UCSF. Specimens were scanned using a cone beam computed tomography unit (CBCT). Biomechanical testing and imaging of the human implant-bone and tooth-bone complexes were performed using a 500N Nano Tomography tensile/compression stage (Deben UK Limited) along with a micro X-ray computed tomography unit (µ-XCT) (ZEISS Xradia 510 Versa). X-ray tomograms were obtained before and after loading the specimens. In the case of human specimens, X-ray tomograms were digitally segmented to determine contact area between bone and implant using FEI's Avizo 3D analysis software. In the case of rat specimens, similar analysis was performed to determine the structural variation of blood vessels within the PDL. Visualisation of the radiotransparent PDL was enabled through the use of a contrast agent - 1% iodine solution [13, 20].

X-ray based digital volumes of tooth and bone under load

X-ray based digital volumes were post-processed for quantitative analyses of various parameters, including structure, mineral density, and mechanical strains (specifically in bone). In the case of the implant-bone complex, the implant was digitally removed from the alveolar socket and the resulting intensity was used as an input to AVIZO software. The deformation in bone under loading was calculated by correlating digital volumes taken at no load to loaded conditions using DaVis software by LaVision Inc. [23]. Subsequently, the components of the 3D strain tensor were calculated from derivatives of the displacement field. The resulting 3D strain field in the alveolar bone was displayed using a direct volume rendering method in AVIZO software [18].

Histochemistry, Immunofluorescence, and Light Microscopy [19]

The generation of scleraxis green fluorescence protein (Scx-GFP) transgenic reporter mice have been previously described [24]. Mouse mandibles were harvested at 3 weeks and 3 months postnatally, fixed in 1% paraformaldehyde, and demineralised in 0.5M ethylene diaminetetraacetic acid (EDTA, pH 8.0) for 1 to 2 weeks at 4°C. The mandibles retrieved from Scx-GFP mice were embedded and snap frozen as described previously [25]. The mandibles were serially sectioned at 10 µm and the cementum-PDL-bone complex of the 2nd mandibular molar was investigated. Primary antibodies included rabbit anti-mouse CD146 (1:250, AB75769, Abcam) and rat anti-mouse CD31 (1:20, DIA-310, Dianova). Slides were incubated in Alexa Fluor 594 donkey anti-rabbit or Alexa Fluor 647 goat anti-rat secondary antibody (Molecular Probes) and were counterstained with Hoechst 33342. Immunoreactivity was visualised with a Nikon Ti-E wide-field fluorescence microscope.

Scanning electron microscopy and energy dispersive X-ray analysis

Cryofractured surfaces were prepared by mounting the specimens on scanning electron microscope (SEM) stubs with a carbon tape followed by sputtering with gold palladium. The topography of the specimens was examined using an S4300 SEM at energy of 5 keV. To investigate the elemental

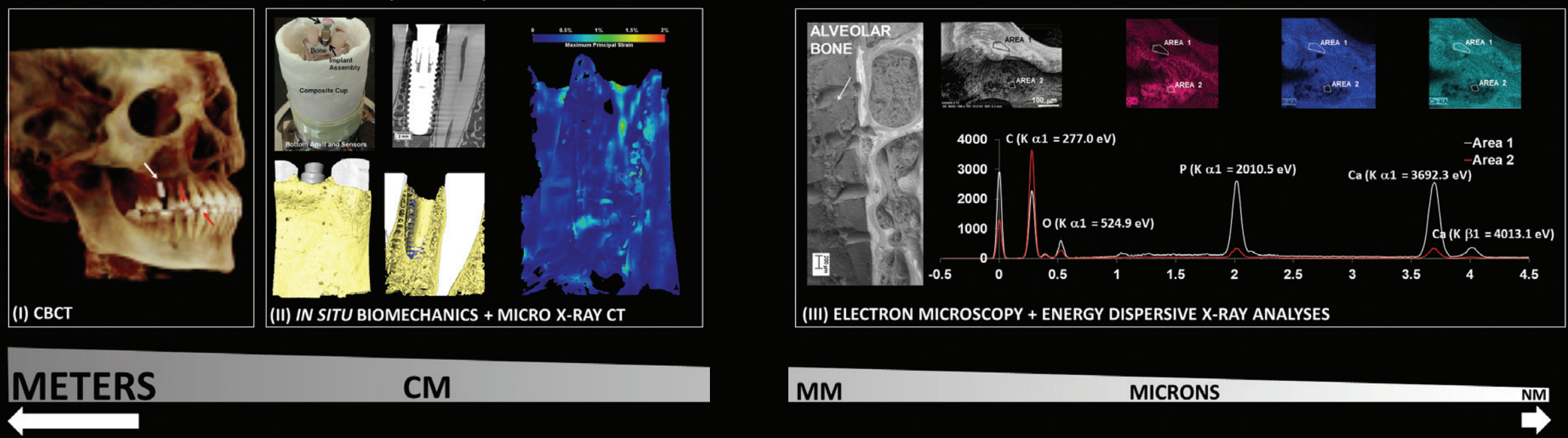

Figure 3. *In situ imaging provides insights into functional osseointegration of implants [left to right]: (I) Patient care X-ray cone beam computed tomography (CBCT) illustrates implant (white arrow) relative to other oral structures including bone, and orthodontic braces (red arrows). (II) In situ loading followed by digital volume correlation of tomograms at no load and loaded conditions revealed contact regions and areas of contact and localized strains under ex vivo conditions. (III) Additional examination of the implant-bone contact regions (white arrow) illustrates the variation in elemental distribution including at the regions of contact. CBCT- cone beam computed tomography; CT – computed tomography, C – carbon; P – phosphorus; Ca – calcium; O – oxygen.*

content at the implant-bone contact region, implants were removed and specimens were sectioned into two halves. Elemental mapping was performed with an energy dispersive X-ray spectroscopy (EDS) unit attached to a Zeiss EVO SEM.

Results

Visualisation of soft-hard tissue attachment sites in a tooth-PDL-bone fibrous joint [20, 21] (Figure. 1)

In general, patient care CBCT illustrates the jaw, the location of teeth, and their contact. Imaging at a lower resolution provides critical information related to the need for teeth/implant positioning to regain optimal function (pain free with minimal to zero long-term effects). Imaging at a higher resolution using an X-ray microscope provides information on the form and function relationship between the tooth and the alveolar bony socket. In humans, the bone and tooth are not fused, but rather separated by the PDL (Figure 1 (II) (III)). Examination of stained specimens of the tooth-PDL-bone complex at a higher resolution using µ-XCT illustrated voids representative of blood vessels within the ligament. In addition, further examination of 1-5 µm thick sections of the complex illustrated tissue-specific heterogeneity in mineral densities (Figure 1 (III)). Interestingly, complementary electron microscopy of cryofractured surfaces showed collagen-rich organic regions and intricate integration of the collagenous ligament (Figure 1 (III), PDL-inserts – asterisks) with the harder alveolar bone of the jaw and cementum of the tooth. The tethered regions of the PDL (shown as PDL-inserts (Figure 1 (III))) in bone and cementum exhibited hygroscopicity, indicative of the water-retaining characteristic facilitated by small macromolecules known as proteoglycans [20]. However, further examination at a nanometer scale by acquiring selected area electron diffraction patterns using a transmission electron microscope illustrated mineral within the collagen fibrils at the attachment sites (Figure 1 (III)). Visualisation with X-rays revealed contextual information about structure and chemistry and provided insights into the overall functional roles of observed organic and inorganic constituents.

Deformation of ligament visualised using in situ mechanical testing coupled to an X-ray microscope, and light microscopy [19] (Figure 2)

Often, conditions observed in patients are mimicked in small-scale animals to map biological processes leading to spatiotemporal changes in tissues. These spatiotemporal maps could aid in understanding clinically observed problems in humans. The smaller size of the specimen allows *in situ* biomechanical testing of intact maxillary-mandibular components and ease in handling of specimens. Detailed examination illustrated narrowing and widening of the functional space, thereby compression and tension of the ligament. With the use of high resolution X-ray imaging following X-ray contrast enhancement through the use of iodine, the deformation of the blood vessel in the PDL as related to simulated force can be seen (Figure 2 (II) – white arrows).

Deformation analysis post-in situ mechanical testing in an X-ray microscope [18] (Figure 3)

Post-processing of implant-bone digital volumes at no load and loaded conditions using DaVis and AVIZO Software illustrated the area over which the implant made contact with bone, and deformations within volumes of bone. The measurement of implant-bone contact through image processing of digitally reconstructed volumes revealed 3D implant-bone contact as seen under *in vivo* conditions. Additional examination of the implant-bone contact regions (Figure 3 (III) – white arrow) illustrated a variation in elemental distribution (Areas 1 and 2) as identified by using energy dispersive X-ray spectroscopy (EDS).

Discussion

Physical and chemical properties of tissues sustain functional demands placed upon joints. Biomechanical *in situ* loading coupled with X-ray imaging is a crucial technique for visualising internal structures of intact joints at loads equivalent to *in vivo* conditions.

Biomechanical testing also allows for mapping deformations not limited to harder materials such as bone; it has the capacity to extend to softer tissues and their interfaces with harder tissues. Radiopaque materials are at an advantage because the structure-function relationship can be investigated with minimum perturbations to the experimental setup. However, for radiotransparent materials such as the PDL, the use of a contrast agent could limit biomechanical measurements and potentially lead to erroneous deformation patterns. Additionally, the size of the specimen is critical from the perspective of joint biomechanics and is limited to the field of view offered by the lens and the size of the loading unit that is housed in the μ-XCT. Regardless, the methodologies reviewed in this article can be used to investigate clinical complications, and can provide insights from which future improvements in surgical planning/techniques can be formulated, thereby improving patient care. These results provided insights into osseointegration evaluated at micro- or nano-levels when investigated in 3D space within the context of function. Additionally, results indicated that implant stability depends on the area and location of implant-bone contacts, and thereby adaptation in bone over time defines the overall functional integration of implant with bone. In general, implant-bone contact is measured using histology, where observations are often limited to the sectioned planes. The measurement of implant-bone contact under load through image processing of digitally reconstructed volumes minimised challenges often met in traditional histology, and revealed the 3D implant-bone contact as seen under *in vivo* conditions.

Deleterious loads can result from clinical interventions in scenarios including the use of dental implants (Figure 3 (I) – white arrow) and orthodontic braces (Figure 3 (I) – red arrows), which are ironically engaged to regain optimal chewing otherwise impaired due to disease or malocclusion. This provides the basis to question the effect of deformation on differentiation of progenitor (stem-like potential) cells, which can be further interrogated and correlated with matrix molecule expressions visualised by using high resolution light microscopy of fluorescently immunolabelled tissues (Figure 2 (III)).

Based on the results through multiscale visualisation of various structural components, questions that can be asked include: what is the role of the water-retaining regions at the ligament-bone and ligament-cementum attachment sites/tethered regions [22]? What has prevented the ligament-inserts from mineralising although they are embedded in harder tissues, bone and cementum? What role do they play from a biomechanical perspective? What types of cells and matrix molecules reside at these attachment sites of the tooth-PDL-bone [19]? In summary, with state-of-the-art technology, contextual imaging can provide an insight into nature's intelligence with the hope of enabling the next level of biomimicry through materials research.

Conclusion

We have demonstrated the crucial role of *in situ* load testing coupled to high resolution X-ray microscope to discern joint and tissue functions. Further application of these methods of multiscale biomechanical testing and visualization towards the myriad mysteries of joint and tissue structure and function will reveal new challenges and questions to be explored.

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Her laboratory has a strong focus on biomechanics and biomineralization with an emphasis on spatiotemporal mapping of "mechano-responsiveness" of tissues and their interfaces. This is done by identifying mechanical strain induced biological processes at soft-hard tissue interfaces (ligament-bone and ligament-cementum interfaces) of the tooth-PDL-bone complex and other organ systems. One of her current research interests involves investigating load-mediated adaptation mechanisms, and to further exploit the biomechanical and mechanobiological concepts for the purpose of tissue regeneration.