Published in final edited form as:

Bone. 2013 June ; 54(2): 285–295. doi:10.1016/j.bone.2013.01.004.

Studying osteocytes within their environment

Duncan J. Webster¹, Philipp Schneider¹, Sarah L. Dallas², and Ralph Müller^{1,*}

¹Institute for Biomechanics, ETH Zurich, Zurich, Switzerland ²School of Dentistry, Department of Oral Biology, University of Missouri, Kansas City, MO, USA

Abstract

It is widely hypothesized that osteocytes are the mechano-sensors residing in bone's mineralized matrix which control load induced bone adaptation. Owing to their inaccessibility it has proved challenging to generate quantitative *in vivo* experimental data which supports this hypothesis. Recent advances in *in situ* imaging, both in non-living and living specimens, have provided new insights into the role of osteocytes in the skeleton. Combined with the retrieval of biochemical information from mechanically stimulated osteocytes using *in vivo* models, quantitative experimental data is now becoming available which is leading to a more accurate understanding of osteocyte function. With this in mind, here we review i) State of the art *ex vivo* imaging modalities which are able to precisely capture osteocyte micro and ultrastructure in 3D, ii) Live cell imaging techniques which are able to track structural morphology and cellular differentiation in both space and time, iii) *In vivo* models which when combined with the latest biochemical assays and microfluidic imaging techniques can provide further insight on the biological function of osteocytes.

Keywords

Osteocytes; high resolution imaging; live cell imaging; microstructure; gene expression; in vivo models

Introduction

Osteocytes represent the terminally differentiated state of the osteoblast lineage and are embedded within the mineralized bone matrix. Because they are trapped within a mineralized “prison”, osteocytes are not easily accessible and therefore our understanding of their role in bone remodeling remains incomplete. Advanced imaging techniques (*ex vivo* and *in vivo*) and the exploitation *in vivo* models to extract quantitative biochemical information are tools which are beginning to provide more clues about both the anatomy and biology of osteocytes, respectively. Synthesis of these data will therefore greatly facilitate a more complete understanding of the osteocyte's function.

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*Corresponding Author Prof. Dr. Ralph Müller, ETH Zürich, Institute for Biomechanics, Wolfgang-Pauli-Strasse 10, 8093 Zürich, Switzerland, tel/fax +41.44.632.4592/1214, ram@ethz.ch, <http://www.biomech.ethz.ch>. All four authors had an equal contribution to the article.

Conflict of interest

The authors declare no conflicts of interest.

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Ex vivo imaging of osteocytes has proved challenging due to the need to develop methodologies for imaging and sectioning of undecalcified specimens or to develop protocols for decalcifying specimens to enable conventional sectioning and imaging techniques to be used. Early imaging approaches relied mainly on staining of the lacuno-canalicular network (LCN) rather than the osteocyte itself using histological stains combined with conventional light microscopy. With the advent of confocal imaging approaches it has become relatively straightforward to image osteocytes and their lacuno-canalicular system three-dimensionally (3D) *in situ* within their bone environment. The recent availability of high resolution micro-computed tomography (CT) systems and synchrotron radiation-based CT has now made it possible to image osteocyte lacunae non-invasively and in a 3D fashion. Standard scanning electron microscopy (SEM) and transmission microscopy (TEM) approaches as well as relief casting of the LCN have facilitated a more detailed analysis of the osteocyte network and the LCN and the more recent use of approaches such as block face sectioning have added 3D capabilities to TEM-based imaging approaches.

In addition to high resolution imaging of osteocytes in fixed or post mortem specimens, transgenic mouse lines have been developed which express fluorescent reporters for the osteocyte lineage. These have provided powerful new tools to enable the imaging of osteocytes *in situ* within living bone specimens as well as to track the differentiation of osteocytes in living cell culture models.

The insight into biological function provided by *in situ* imaging can be greatly enhanced via the use of *in vivo* loading models with advanced quantitative biochemical assays in an approach termed ‘microfluidic imaging’. This article will review the wide variety of imaging modalities that are now available to study osteocytes *in situ* (both *ex vivo* and *in vivo*). Furthermore, the use of *in vivo* models and microfluidic imaging will also be discussed. In each case the advantages and limitations of these tools will be addressed.

Studying osteocytes *ex vivo* using high resolution imaging

Light microscopy

There is more than a century of tradition in studying intracortical bone microstructure, such as Haversian canals, osteocyte lacunae, and canaliculi. During the early days of investigations into bone microstructure, histological sectioning in combination with light microscopy was the predominant imaging approach. The first bone preparation protocols for the assessment of the intracortical microstructure were developed during the first half of the last century, including the use of basic stains such as Alizarin red or basic fuchsin. These techniques stain the lacuna-canalicular system rather than the osteocytes themselves, but have proved very useful in revealing the intricate network of canaliculi throughout the bone matrix and the interconnectivity of osteocytes. These protocols were refined later in the very early work of Frost at the end of the 1950’s and at the beginning of the 1960’s, where the intracortical bone microstructure was investigated in detail [1]. In more recent contributions from the 1980’s and 1990’s, researchers at the University of Modena, Italy extensively used light microscopy to study the lacuno-canalicular network (LCN), i.e. the osteocyte lacunae and their interconnected canaliculi. These studies specifically addressed correlations between the local LCN extension and the metabolic activity of osteoblasts and osteoclasts, while the functional interplay between the activity of osteocytes and other bone cells could not be answered conclusively [2]. However, their studies supported the notion that osteocytes are critically involved in the regulation of bone homeostasis, namely through their postulated potential to act as mechanosensors [3].

Scanning electron microscopy (SEM)

With the wide availability of scanning electron microscopes (SEM) in the mid-1960's, the intracortical and intratrabecular bone microstructure became accessible to a broader researcher community and could be imaged at resolutions beyond the diffraction limit of visible light at a few hundred nanometers. This allowed visualization of canaliculi with diameters on the same scale, i.e. a few hundred nanometers as shown for example in [4], where canalicular numbers were derived from measurements based on light microscopy and SEM. Casting protocols for SEM imaging originally developed to display the microstructure of dentin were adapted to image the LCN within cortical bone and more recently they were further developed [5] (Figure 1A). Nonetheless, the basic imaging principle remained essentially the same, namely to present a replica of the LCN using SEM after complete or partial acid-etching of the mineralized bone matrix. In their study on the role of osteocytes in mineral metabolism, Feng *et al.* [6] showed that loss of dentin matrix protein (DMP1), which is substantially expressed in osteocytes, causes rickets and osteomalacia. Moreover, using SEM images of acid-etched bone samples from *Dmp1*-null mice abnormalities in the distribution and organization of the LCN system were reported, which are due to *Dmp1* ablation.

Confocal microscopy

Another approach to image the intracortical and intratrabecular bone microstructure and cellular structure is confocal microscopy, whose principles were developed in the 1950's and whose first applications on bone tissue were published in the mid 1980's. In contrast to inherently two-dimensional (2D) imaging techniques such as light microscopy and SEM, in confocal microscopy, optical sections at different focal planes can be stacked together to generate a three-dimensional (3D) representation of the sample under investigation. Endogenous (auto)fluorescence of the bone tissue can be used to provide contrast for confocal microscopy measurements of the LCN. More often, various fluorescent staining agents are used in conjunction with modern confocal laser scanning microscopy (CLSM), such as rhodamine and fluorescein, which can be incubated with undecalcified bone sections and will be taken up into the LCN [7]. More specific staining agents, such as fluorescein isothiocyanate (FITC)-conjugated phalloidin and DAPI, label the actin skeleton of osteocytes and/or the DNA of their cell nucleus in such a way that the components of the osteocyte network can be directly imaged [8] and separately displayed in 3D [9] (Figure 2). This provides an image of the cellular structures themselves, in contrast to the SEM assessment of the LCN, which represents a negative imprint of the mineralized bone matrix only. CLSM has been used specifically to demonstrate the correlation between the organization of the osteocyte network and the collagen orientation [10], which is important for bone mechanics. Moreover, differences in osteocyte network morphology have been observed by CLSM for different bones [9] and various bone pathologies [11] and they have been suggested to reflect an adaptation to different loading conditions. At the same time, distinct osteocyte network morphologies have been proposed to be related to differences in osteocyte mechanosensitivity, which is crucial for bone health.

X-ray computed tomography (CT)

A major drawback with CLSM is the limited maximum focal plane depth of around 100-150 μm . Additionally, CLSM is tainted with image artifacts, such as signal attenuation with increasing focal plane depth or aberrations due to refractive index mismatch. These artifacts are practically absent in (conventional) X-ray absorption-based computed tomography (CT). The introduction of micro-computed CT (μCT) desktop scanners in the mid 1990's along with the development of 3D morphometric measures to quantify trabecular microarchitecture laid the foundations for μCT to become a standard for bone morphometry. In bone research, the standard application of desktop μCT systems with typical voxel sizes

in the order of 5-100 μm was – and still is – the basis for quantitative characterization of whole bone geometry and trabecular microarchitecture. On the other hand, synchrotron radiation-based CT (SR CT) was introduced to image the intracortical and intratrabecular bone microstructure in the late 1990's [12], and was further developed and applied later to investigate the intracortical canal network (living space of the vasculature and/or bone remodeling units), specifically by the group of Peyrin [13], by Cooper *et al.* [14], and by Schneider *et al.* [15], as well as to study osteocyte lacunae within trabecular [12] and cortical bone [15] (Figure 3). Quite recently, Pacureanu *et al.* devised an optimized imaging protocol for SR CT [16] and pushed the spatial resolution closer to the diffraction limit of visible light at a few hundred nanometers, with the result that on top of osteocyte lacunae, larger canaliculi could be distinguished in the human femoral mid-diaphysis. However, a limitation of this approach is that segmented canaliculi from these measurements were discontinuous since spatial resolution was comparable to the range of typical canaliculi diameters. It is only recently that desktop μCT scanners have become available on the market with voxel sizes below 1 μm . These have allowed the assessment of osteocyte lacunar morphology and alignment in different mouse [17] and human bones [11]. In addition, another group examined mean osteocyte lacuna volume and lacuna distribution in human transiliac crest [18], further explored the influence of menopause on mean lacuna volume at the same site [19], and they eventually analyzed the impact of parathyroid hormone (PTH) on lacuna density and volume in a rat model for osteoporosis [20].

Transmission X-ray microscopy (TXM)

An alternative imaging approach to overcome the resolution limitation of conventional desktop CT and SR CT, which is limited by the diffraction limit of visible light on the image detector, is to build a transmission (full-field hard) X-ray microscope (TXM), where a zone plate acts as an X-ray lens and magnifies the image of the sample onto the detector, similar to the objective in a traditional light microscope. The Rayleigh resolution of a zone plate TXM system is determined by approximately the size of the outermost smallest zone width, and thus, is tightly connected to advancements in the lithographic fabrication process of zone plates, currently allowing hard X-ray microscopy resolutions well below 50 nm. Whereas SR-based zone-plate TXM setups are frequently used in 2D, as well as in 3D when combined with a rotation stage for tomography, it was not until recently that a first desktop TXM CT system was implemented [21], which is operated with a commercial X-ray tube. An initial TXM CT measurement performed on this system provided a 3D reconstruction of an osteocyte lacunae and radiating canaliculi of a tibial trabecula in the mouse [22]. Although the spatial resolution of the system in 2D has been reported to be below 50 nm [22], canaliculi in the 3D reconstructions were interrupted. Therefore further refinements to this technology are needed in order to accurately model the canaliculi in 3D.

Transmission electron microscopy (TEM)

Higher spatial resolutions can be achieved using electrons instead of X-rays, where the resolution of an electron microscope increases in a manner that is inversely proportional to the square root of the applied voltage, and is typically in the nanometer range. TEM has been extensively used to investigate in 2D the ultrastructure of osteoblasts and osteocytes including their dendritic processes. The morphology of osteocytes and their processes were further characterized in 3D by successive serial sectioning and TEM imaging [23]. More recently, Kamioka *et al.* adopted TEM computed tomography (TEM CT) on an ultra-high voltage electron microscope, where silver-stained osteocytes in 3- μm chick calvaria sections were assessed at an accelerating voltage of 2 MeV and at a nominal resolution of 16 nm [24]. Prominent silver deposition for young osteocytes, which has been observed in their nuclei and in the pericellular space, was used to segment the cell nuclei, bodies, and the osteocyte processes (Figure 1B). Kamioka and colleagues found that the surface of the

osteocyte network was irregular and that the size and shape of the cell processes varied significantly. Besides the demanding sample preparation, a major problem of TEM is the fact that for a dense material like bone, even at ultra-high voltages, the maximal sample thickness that can be penetrated by electrons is only a few μm due to strong scattering and absorption for thicker specimens.

Ptychographic computed tomography (CT)

A fundamentally different CT imaging strategy beyond the diffraction limit of visible light is motivated by the idea to completely remove any lens in the system, in order to perform coherent diffraction imaging (CDI) or lensless imaging [25], which is theoretically limited by the used radiation only, i.e. around 1 Å for hard X-rays. Ptychographic CDI is an emerging iterative phase retrieval method with no fundamental limitation in sample size, which provides the complex sample transmission function. Ptychographic CDI has recently been combined with a CT setup for ptychographic (X-ray) CT, where the LCN of the femoral mid-diaphysis in the mouse has been retrieved at an isotropic voxel size of 65 nm [26], offering a continuous representation of individual canaliculi. In addition to the reconstructed LCN morphology, the local mineral density was simultaneously reconstructed in the same experiment by ptychographic CT in terms of (absolute) electron density with fluctuations of less than 0.2% corresponding to less than 5 mg/cm^3 in mass density.

Serial focused ion beam/scanning electron microscopy (FIB/SEM)

A key problem, which is common to all the CT-based techniques described above, is their limited field of view (FOV) which adversely affects the assessment of larger tissue volumes, containing for example a representative segment of the osteocyte network and/or the LCN. One concept to overcome this limitation in 3D at a sufficiently high resolution is the strategy to go back to the elementary direct imaging method of consecutive physical probe sectioning and imaging, similar to conventional histology based on light microscopy. However, the imaging approach must have improved spatial resolution compared to light microscopy and it must be automated in order to resolve the intracortical and intratrabecular bone microstructure in a relevant volume. One implementation of this concept is serial focused ion beam/SEM (FIB/SEM). In serial FIB/SEM, several thin sections in the 10 nm range are milled away from the sample's block face using a focused ion beam, which replaces the diamond knife for mechanical cutting in traditional histology. These sections are then scanned by SEM. When applied in a serial and automated fashion, a 3D reconstruction of the specimen can be generated at EM resolution. FIB systems have been mostly used in materials science and in the semiconductor industry since the early 1980's, and the application of serial FIB/SEM has broadened with the automation of the dual beam FIB/SEM imaging process in the mid-2000's, including research fields in the life sciences, especially in the neurosciences [27]. Regarding hard tissue characterization, serial FIB/SEM has been broadly employed to study dental/implant interfaces and bone/implant interfaces. Moreover, Earl *et al.* lately examined the intradental tubule network, including major, fine, and microbranches from the micrometer range down to several hundred nanometers diameters [28], similar to the dimensions of the canaliculi in bone. The first attempt to image the LCN in bone goes back to Stokes *et al.* [29], where the representation of the canaliculi (species not specified) was fragmentary only. More recently, Schneider *et al.* presented for the first time a serial FIB/SEM data set, with interconnected osteocyte lacunae and continuous canaliculi (Figure 1C), including quantitative morphometry of the LCN, which was assessed at a nominal resolution below 30 nm in-plane and between serial sections of the femoral mid-diaphysis in the mouse [30].

Serial block-face scanning electron microscopy (SBF SEM)

The more traditional approach in EM, which tackles the problem of a limited FOV in CT-based techniques, is the method of successive serial sectioning with an ultramicrotome for individual sections, which are then imaged using TEM. However, this procedure cannot be easily automated for imaging of an extended tissue volume. Moreover, registration of such serial sections could introduce image artifacts. This is the reason why serial block-face scanning EM has been realized exclusively for SEM (SBF SEM). The first SBF SEM setup was put into practice by Leighton in the early 1980's, who built a miniature microtome, which was operated remotely in a standard SEM [31]. SBF SEM was revisited in the mid-2000's by Denk and Horstmann who developed a diamond-knife ultramicrotome, sectioning inside the chamber of an SEM [32], which was subsequently automated further and commercialized [33]. The main application field of SBF SEM is currently in the neurosciences [34], where neuron morphologies from extended SBF SEM image stacks are extracted. Automated SBF SEM has not been applied so far to study the intracortical and intratrabeular microstructure, but would offer an efficient way to image the intracortical and intratrabeular microstructure of bone in 3D for an extended FOV, or even for a whole bone. These types of experiments are already well advanced in the field of neuroscience, where researchers envisage possible experimental setups to assess all neural connections or the complete brain connectivity, called the connectome, based on SBF SEM. In the future, we may therefore be able to assess the entire osteocyte network and/or the whole LCN of a full bone, which would have a significant impact in investigations, where cell-cell communications in bone are studied.

Studying osteocytes *in situ* using live cell imaging

Advantages and limitations of live cell imaging approaches

Over the past two decades or so, technologies for imaging of living cells using light and confocal microscopy have advanced at a rapid rate. This, coupled with the discovery of green fluorescent proteins (GFPs) and their derivatives (reviewed in [35]) and the development of a seemingly limitless array of fluorescent imaging probes and GFP-fusion proteins, has made it possible to image almost any intracellular or extracellular structure or protein in living cells and tissues (reviewed in [36]). A large selection of fluorescent probes and reagents are commercially available to the researcher for investigating biological events in living cells, including fluorescent antibodies, kits for fluorescently labeling proteins of interest, dyes for cell and nuclear tracking, probes for labeling of membranes and organelles, fluorescence reagents for determining cell viability, probes for assessing pH and ion flux and probes for monitoring enzyme activity, etc. In addition, a variety of GFP-derived fluorescent protein vectors are available that can either be used as reporter constructs or to generate fusion constructs with a protein of interest. These enable the live monitoring of gene expression and protein localization *in vivo*, and in real time.

The traditional approach of collecting “static” images of fixed or post mortem cells and tissues provides a snapshot view of events at a single fixed point in time. However, this inherently overlooks the dynamic aspects of the biology being examined. In contrast, live cell imaging enables the visualization of temporal changes in living specimens and can reveal novel aspects of the biology that may not otherwise have been appreciated. Additionally, the datasets generated from time-lapse imaging are information rich and can be interrogated quantitatively to enable measurement of cellular, subcellular and tissue dynamic events as a function of time (reviewed in [37]).

Although these approaches are leading to exciting discoveries that are advancing our understanding of biological systems, there are several limitations that need to be acknowledged. Firstly, the use of any fluorescent probe has the potential to perturb or alter

the biology being examined and this must always be taken into account when interpreting live imaging data. For example, fusion of GFP sequences, which are approximately 27kDa in size, with the protein of interest may disrupt the normal function of the protein. Therefore, validation studies are needed to make sure that the fusion protein still functions similarly to the wild type form. It is also advantageous to confirm findings with more than one type of imaging probe if possible. For example, a GFP fusion protein can be used for *in vivo* localization of a specific protein and key data can be confirmed using a fluorescence-conjugated antibody against the same protein. When developing live cell imaging protocols, there is always a compromise between obtaining a high enough signal-to-noise ratio to enable quantitative measurements and to obtain sufficient image resolution, while at the same time avoiding phototoxic effects to the cells (reviewed in [36, 38]). Therefore, to ensure cell viability, the researcher may have to accept a lower image quality and resolution than would be acceptable for equivalent images of fixed specimens. Light microscopy based live cell imaging approaches that use widefield or confocal microscopy are also limited by issues such as signal attenuation with depth of penetration into the tissue, as mentioned earlier in this review. For a more extensive discussion of the advantages and limitations of live cell imaging methods in relation to imaging of bone cells, please refer to Dallas and Veno 2012 [36]. Technologies such as multiphoton fluorescence microscopy can increase the depth of tissue penetration for live cell imaging applications and reduce phototoxicity by using a longer wavelength light to excite the fluorophores. These instruments are becoming more widely used for live imaging applications due to their advantages over conventional widefield and confocal microscopy systems (reviewed in [39]).

Live cell imaging approaches as applied to osteocytes

Recently, live cell imaging approaches have been applied to the study of osteocytes. The development by Kalajzic *et al* of transgenic mice expressing the GFP^{topaz} reporter variant under control of the osteocyte-selective dentin matrix protein-1 (Dmp1) promoter [40] has underpinned such studies of osteocytes *in situ* within their environment. Organ cultures of neonatal calvaria from these mice have provided a useful model for imaging the dynamic properties of osteocytes [36, 41-43]. Another way in which this model can be used for imaging osteocyte dynamics is by using long term cultures of primary osteoblasts isolated from these mice [36, 42, 44]. These cells differentiate when cultured under mineralizing conditions to form mineralized nodules in which the transition to the osteocyte-like phenotype can be monitored by GFP expression. To gain maximum information, imaging of the GFP reporter can be combined with other fluorescent probes, such as alizarin red to monitor mineral deposition. The mice can also be crossed with other transgenic reporter lines, for example mouse lines in which the osteoblasts are tagged with GFP^{cyan} [45].

The old view of the osteocyte was as an immobilized, inactive cell. However, live imaging of osteocytes in neonatal calvarial organ cultures or primary mineralizing bone cell cultures from Dmp1-GFP transgenic mice has shown that osteocytes may actually have dynamic properties that were not previously appreciated [36, 41-43]. These studies have revealed that the dendritic connections between osteocytes may not be permanent but rather the dendrites are repeatedly extended and retracted (Figure 4). Transient dendritic connections appeared to be made between adjacent osteocytes and the osteocytes also showed deformations/undulating motions of their cell bodies within their lacunae, suggesting that even though they are entrapped within a lacuna, they remain active and still exhibit motile properties [43, 46]. The deformations that the osteocyte cell body undergoes within its lacunae were measured and averaged around 3% but could be as high as 12%. One implication from this is that the strains experienced by an osteocyte within its lacuna when bone is mechanically loaded may be dependent not only on the material properties of the bone itself but also potentially on the configuration of the osteocyte within its lacuna.

The more recent development of transgenic mice expressing a membrane targeted GFP variant selectively in osteocytes has provided a new tool for more precise imaging of osteocytes and their dendritic processes/membrane dynamics in living bone [46]. Time-lapse imaging studies in neonatal calvarial organ cultures and primary mineralizing osteoblast cultures from these mice have confirmed the motions of the osteocyte cell body and dendrites and suggested extensive ruffling of the osteocyte cell membrane within its lacuna. They have also shown that osteocytes may shed membrane-bound vesicle-like structures from their cell body and dendrites [46]. The function of these vesicles is currently unclear. They may provide a mechanism for reduction of osteocyte cytoplasmic volume. Alternatively, they may regulate mineral deposition and/or may provide a mechanism for intercellular communication through delivery of messenger RNA and proteins to the target cells, as has been described for microvesicles in other cell systems (reviewed in [47, 48]).

A surprising finding from live imaging studies of osteocytes in neonatal calvarial organ cultures was the observation of a subpopulation of motile cells on the bone surface that express the Dmp1-GFP transgene but exhibit a polygonal non-dendritic morphology [43, 46]. It was shown that these surface motile Dmp1-GFP positive cells also express the early osteocyte marker E11/gp38, suggesting that they may represent a precursor that is already committed to becoming an osteocyte [43].

Time-lapse imaging studies in mineralizing osteoblast cultures have revealed that the kinetics of Dmp1-GFP expression and mineralization are integrated, with clusters of motile cells first switching on Dmp1-GFP expression followed by mineral deposition [42, 44] (Figure 5). These Dmp1-GFP positive cells also express E11/gp38, suggesting that they are transitioning towards the osteocyte phenotype. Deposition of mineral was found to be associated with an arrest in motility of the Dmp1-GFP positive cells and a change in morphology from a polygonal to a highly dendritic morphology, characteristic of osteocytes. The data suggest that the processes of osteocyte differentiation and mineralization are tightly integrated and that the cell type responsible for mineralization is a cell that is already transitioning towards the osteocyte phenotype.

Recently, Ishihara et al have used time-lapse imaging approaches to image calcium signaling oscillations in living osteocytes in embryonic chick calvaria [49] (Figure 6). Their studies showed that osteoblasts and osteocytes show oscillations in intracellular calcium concentrations and that calcium release from intracellular stores plays a key role in these calcium oscillations. In osteocytes but not osteoblasts, gap junctional communication appeared to be important for maintenance of the calcium oscillations. Such studies are an important advance, as prior to this work, intracellular calcium signaling has been reported from *in vitro* studies of osteocytes and was thought to be important in mechanotransduction [50-52]. However, it was not known whether these phenomena actually occur in osteocytes *in situ* within their mineralized lacunae.

Live cell imaging studies as applied to investigating osteocyte biology are still in their infancy. In addition to revealing the dynamic properties of osteocytes, revealing the dynamics of intracellular signaling pathways, such as calcium oscillations, monitoring the temporal integration of osteocyte differentiation and mineralization and determining what actually happens during the osteocyte embedding process, live imaging studies have considerable potential to address many as yet unresolved questions in osteocyte biology. For instance, is osteocyte differentiation an irreversible process or can the osteocyte dedifferentiate back into an osteoblast when it is released from its lacuna? What is the fate of the osteocyte after osteoclastic resorption? Do osteocytes make dendritic contacts with cells in the marrow and vasculature? With the rapid advancement of imaging technologies

and the development of more and more sophisticated fluorescent reporters, there is no doubt that some of these questions will be answered in the very near future.

Studying osteocytes *in vivo* using gene expression analysis

Owing to the fact that osteocytes are deeply embedded in hard mineralized tissue they are less accessible compared to other cells types. As a result *in vivo*, biochemical data characterizing their precise role in bone remodeling remains limited. A number of *in vivo* models have been developed to study their function. These models typically harvest large osteocyte populations and employ technologies which provide a comprehensive assessment of a large number of genes which are both up-regulated and down-regulated in response mechanical stimulation. In this section we provide an overview of these models and highlight the strategies and new technologies which could be employed to further enhance our understanding of the osteocyte.

Global gene expression of load induced bone adaptation

To comprehensively assess osteocyte gene expression in a mouse model for load induced bone adaptation, current state-of-the-art approaches extract large populations of osteocytes from loaded bone and perform micro-array-analysis to quantify the expression levels of tens of thousands of different genes. Using this technology, probable molecular networks describing osteocytes function and interactions with other cell types are constructed. This is achieved via the use of data mining techniques to search literature pertaining to relevant genes/proteins together with various statistical algorithms. For example, using the recently established mouse tail loading model [53] Wassermann et al [54] dynamically loaded the sixth caudal vertebra (C6) of C57BL/6 (B6) mice and harvested a large number of osteocytes (> 10,000) from mechanically stimulated trabecular bone. Following isolation of high quality mRNA from osteocytes and the application of micro-array-analysis, patterns of gene expression were quantified for short and extended periods of loading. Analysis of 34,000 different genes revealed that hundreds of genes were differentially expressed [55]. Comparison of global osteocyte gene expression between sham-loaded and loaded groups for a single bout of loading revealed a total of 287 up-regulated and 52 down-regulated genes. Similarly for the chronic loading regime a total of 778 genes were found to be up-regulated compared to the 561 which were down-regulated. Amongst these were genes which have known or suspected roles in osteocyte metabolism as well as genes encoding extracellular proteins which potentially facilitate communication with both osteoblasts and osteoclasts.

The vertebra loading model is not the only model which has been established to investigate load induced bone adaptation. A number of different animal loading models have been established which focus more on the response of cortical bone to mechanical stimulation. These include ulna [56], tibia [57] and femoral [58] loading models. Some of these models have also been used as part of global gene expression studies similar to that described for the mouse-tail loading model [59-61], the main difference being that instead of isolating pure osteocyte cell fractions, mRNA from the entire heterogeneous cell population contained within the loaded bone had been pooled and assayed (i.e. osteoblasts, osteoclasts, stromal cells).

Microfluidic imaging of local gene expression for load induced bone adaptation

Global gene expression assays derived from *in vivo* models for bone adaptation have identified a number of candidate genes and revealed potential load regulated pathways. However, caution must be exercised when interpreting these data. The harvesting and analysis of large populations of osteocytes reports gene expression averaged over tens of

thousands of cells, each of which reside in different micro-environments characterized by different levels of mechanical strain and local osteoblastic/osteoclastic activity. It is therefore possible that key genes and networks are being concealed. Recently a few studies [62, 63] have begun to investigate local regulation of gene expression in osteocytes by comparing 2D histology sections from loaded bone stained for specific molecular targets (sclerostin) with micro finite element (μ FE) models. Whilst informative, these approaches are still very much qualitative and only permit the analysis of one specific molecular target at a time. To overcome these limitations a novel combination of old and new technologies has recently been proposed (termed microfluidic imaging) which promises to map, quantitatively, and in three dimensions the expression of multiple genes in individual osteocytes. This ‘microfluidic imaging’ approach is reviewed in more detail elsewhere [64] but can be briefly described by the following workflow (Figure 7): 1) Bone formation and resorption are spatially mapped and quantified in a mouse loading model using *in vivo* μ CT [65] and 3D image registration techniques [66]. 2) The micromechanical environment in loaded bone is determined by creating μ FE models of the loaded bone from the initial CT image [67, 68]. 3) At the end of a specific loading regime, cryosectioning [69] and laser-capture-microdissection technologies are used to extract individual osteocytes [70] which are then processed (dna-micro-arrays, RT-PCR) using state-of-the-art lab-on-a-chip technologies [71]. 4) By registering 2D images of cryosections with registered μ CT images and their μ FE models osteocyte positions along with their unique expression profiles are mapped back to their original *in vivo* micro-environments. It is anticipated that the vast amount of data generated using this approach can be used to build, feed and validate computational models of bone which incorporate all of the different length scales, from the organ-level to the cellular-level [64, 72]. By combining computational and experimental approaches in this way it is hoped that the move towards a more complete understanding of the osteocyte and bone biology in general will be expedited.

The current state of our knowledge regarding the molecular mechanisms which underpin the mechanical response of bone is at best fragmented. The Wnt/ β -catenin signaling pathway [73, 74] has received much attention and is now recognized as an important regulator of bone mass and bone cell function, however it still remains to be determined how this pathway interacts with other key molecular components, which include RANKL, sclerostin [75-77], nitric oxide [78], prostaglandin [79] and the many others identified in the *in vivo* loading studies presented here. Whilst *In vivo* models exploiting comprehensive gene expression tools may have identified a number of candidate genes/proteins how these different elements interact remains a probabilistic construct. If definitive answers are to be found synergistic approaches will be required using the technologies discussed here. In summary, advanced techniques for imaging osteocytes *ex vivo*, *in situ*, and *in vivo* combined with localized quantification of gene expression will be key to unraveling the function of these fascinating cells. Factor into this the emerging field of multiscale computational modeling and it becomes clear that the tools are now at our disposal to significantly enhance our understanding of osteocytes in bone biology.

Acknowledgments

Authors gratefully acknowledge funding from SystemsX.ch (2010_071, DJW/RM), the Swiss National Science Foundation (SNF 205321_132779, PS/RM) and the National Institutes of Health (R21-AR054449, RO1-AR051517 and PO1-AR46798, SLD).

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Highlights

1. Comprehensive review on studying osteocytes ex vivo using high resolution imaging
2. Comprehensive review on studying osteocytes in situ using live cell imaging
3. Comprehensive review on studying osteocytes in vivo using gene expression analysis

Figure 1.

Osteocyte network and lacuno-canalicular network (LCN) retrieved from different high resolution *ex vivo* imaging techniques. (A) Corrosion casting replica of the LCN in cortical mouse bone. The data have been assessed by scanning electron microscopy (SEM). Figure reprinted from [5] with permission. (B) Osteocyte network of chick calvariae. The osteocyte cell bodies and their processes have been assessed from 3- μ m-thick bone sections by transmission electron microscopy computed tomography (TEM CT) at a nominal resolution of 16 μ m. Contrast of the osteocyte network surface for TEM CT imaging was imparted by silver staining, which allowed for the displayed three-dimensional reconstruction of the osteocyte and the cell processes' surface. Figure reprinted from [24] with permission. (C) LCN of the femoral mid-diaphysis in the mouse assessed by serial focused ion beam/scanning electron microscopy (FIB/SEM). For serial FIB/SEM imaging, thin bone layers (down to a few nanometers) are milled away from the sample's block face by an ion beam, alternating with SEM imaging of the block face. The acquired SEM sections are then registered and stacked together for following segmentation of the three-dimensional representation of the LCN, including osteocyte lacunae (yellow ellipsoid portions) and canaliculi (green tubes). The data have been assessed at a nominal resolution of 18.6 nm $\times$ 18.6 nm in-plane and at 29.5 nm between serial sections. Figure reprinted from [30] with permission.

Figure 2. Stained osteocyte network imaged by confocal laser scanning microscopy (CLSM). The images from parietal bone (a, c, e, g) and tibia (b, d, f, h) in the mouse show intensity z-projections of the original CLSM slices (a, b) and surface renderings of osteocyte cell bodies and processes (c, d and e, g), and of osteocyte nuclei. (e, f, g, h). Figure reprinted from [9] with permission.

Figure 3. Intracortical microstructure of femoral mouse bone. The canal network (red tubes) and osteocyte lacunae (yellow ellipsoids) have been obtained from synchrotron radiation-based micro-computed tomography (SR CT) at a voxel size of 700 nm. It is the bone's X-ray absorption through which the intracortical microstructure has been segmented as a negative imprint of the mineralized bone matrix. Figure reprinted from [15] with permission.

Figure 4.

Still frames from a time lapse imaging series of Dmp1-GFP-positive osteocytes in a 7 day old neonatal mouse calvarial explant. Images were acquired every 20 min for 12 h on a widefield fluorescence microscope using the green fluorescence channel for GFP (shown) and DIC for imaging of the bone explant (not shown). The osteocytes marked with an asterisk (*) show motions of their dendrites, which adopt various configurations at different times during the time lapse imaging period. Below each image, the outline of these osteocyte and their dendrites is traced to illustrate the dendrite conformations. Bar = 30 μ m. Modified from [36] with permission.

Figure 5.

a) Still frames from a time lapse imaging series of mineralizing primary osteoblast cultures from Dmp1-GFP transgenic mice in which alizarin red was used as a vital dye for mineral deposition. The movie was started when clusters of GFP positive cells had formed in the cultures and images were acquired every 20 min for 48 h in DIC and two fluorescent channels. Triple merged images of DIC, GFP and alizarin red are shown. Bar = 100 μm. b) Quantitation of mineralization dynamics from the time lapse sequence shown in (a). Mineralization was quantified using Image J software by thresholding of alizarin red image stacks followed by measurement of the mineralized area (red line). The number of GFP-positive cells were counted (green line). Note the deposition of mineral beginning 10 h after addition of 4 mM β-glycerophosphate and increasing until 40 h. Mineral deposition occurs specifically where there are clusters of GFP-positive cells and mineralization is accompanied by an increase in the number of GFP-positive cells. These data suggest that the cells responsible for mineral deposition are already transitioning towards the osteocyte phenotype. Figure reprinted from [36] with permission.

Figure 6.

Time lapse imaging of the autonomic $[Ca^{2+}]_i$ responses of osteocytes in intact calvarial explants (A) Fluorescence and pseudocolor images of living osteocytes in intact calvarial explants. The color scale represents the relative fluorescence intensity. The numbered cells in the fluorescent image correspond to the cells assessed in panel B. (B) representative normalized relative fluorescence intensity plots of individual osteocytes in intact calvarial explants. Each line represents the time course of the $[Ca^{2+}]_i$ response of the individual osteocyte indicated by the corresponding number in panel A. The lower case letters (a-b) in (B) indicate the $[Ca^{2+}]_i$ response during which the frames shown in (C) were taken. (C) serial pseudocolor images of the osteocytes shown in A taken at 0, 99, 162 and 252s after the initiation of monitoring. Consecutive images, which were collected from a single Z plane were taken at 3 second intervals for 5 min. Bar = 10mm. Figure reprinted from [49] with permission.

Figure 7. The Mechanical Systems Biology framework for investigating load-induced bone adaptation is a combined experimental and computational approach which can be separated into 3 different workflows. Figure reprinted from [64] with permission.