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Novel approaches for two and three dimensional multiplexed imaging of osteocytes

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Abstract

Although osteocytes have historically been viewed as quiescent cells, it is now clear that they are highly active cells in bone and play key regulatory roles in diverse skeletal functions, including mechanotransduction, phosphate homeostasis and regulation of osteoblast and osteoclast activity. Three dimensional imaging of embedded osteocytes and their dendritic connections within intact bone specimens can be quite challenging and many of the currently available methods are actually imaging the lacunocanicular network rather than the osteocytes themselves. With the explosion of interest in the field of osteocyte biology, there is an increased need for reliable ways to image these cells in live and fixed bone specimens. Here we report the development of reproducible methods for 2D and 3D imaging of osteocytes in situ using multiplexed imaging approaches in which the osteocyte cell membrane, nucleus, cytoskeleton and extracellular matrix can be imaged simultaneously in various combinations. We also present a new transgenic mouse line expressing a membrane targeted-GFP variant selectively in osteocytes as a novel tool for in situ imaging of osteocytes and their dendrites in fixed or living bone specimens. These methods have been multiplexed with a novel method for labeling of the lacunocanicular network using fixable dextran, which enables aspects of the osteocyte cell structure and lacunocanicular system to be simultaneously imaged. The application of these comprehensive approaches for imaging of osteocytes in situ should advance research into osteocyte biology and function in health and disease.

Keywords

Osteocytes; Osteoblasts; 3D imaging; Bone histology; Confocal imaging; Collagen

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Introduction

Osteocytes have historically been viewed as quiescent cells that are inactive compared to other bone cell types, such as osteoblasts and osteoclasts. However, the last decade has seen these cells come to the forefront of mineralized tissue research to be recognized as a highly active bone cell type that plays multifunctional roles in skeletal homeostasis (reviewed in [1–3]). Osteocytes make up about 90–95% of the cells in adult bone and can live as long as decades in the skeleton. They have a stellate morphology with long cytoplasmic processes (dendrites) extending out from the cell body. They also have a unique location, embedded in bone within a mineralized lacuna, with the dendrites extending through narrow channels in the bone matrix, called canaliculi.

Accumulating research is revealing that, far from being quiescent, osteocytes play key roles in a number of diverse skeletal functions, including mechanosensation, regulation of osteoblast and osteoclast function and regulation of mineral homeostasis (reviewed in [1–5]). Sclerostin, a protein that is highly expressed by osteocytes, has been shown to be a potent negative regulator of bone mass [6] and inhibition of sclerostin has become a major target for development of new bone anabolic therapies for treatment of osteoporosis [7–10]. Osteocytes have also recently been shown to be a major source of receptor activator of nuclear factor kappa-B ligand (RANKL) in bone and to be key players in the regulation of osteoclastic bone resorption [11,12]. Exciting advances in the field over the past decade have shown that osteocytes play an important role in phosphate and calcium homeostasis. They express dentin matrix protein-1 (DMP1) and phosphate regulating endopeptidase homolog, X-linked (PHEX), which control phosphate metabolism through regulation of fibroblast growth factor 23 (FGF23) ([13,14]; and reviewed in [3,5]). Moreover, the osteocyte network actually appears to function as an endocrine organ by secreting FGF23 into the circulation. This FGF23 then acts on the gut and kidney to regulate phosphate uptake and reabsorption. Another mechanism by which osteocytes can regulate ion homeostasis is through remodeling of their perilacunar matrix [15,16], which can release calcium and phosphate from the lacunae and canaliculi into the circulation. Not only do viable osteocytes play multiple roles in diverse skeletal functions, but the dying (apoptotic) osteocyte is also thought to play important regulatory roles in controlling osteoclast activity [17]. Overall, an exciting new paradigm is emerging of the osteocyte as a central orchestrator within the skeleton that may serve to integrate mechanical, hormonal and growth factor signals to regulate bone mass.

Osteocytes have been difficult to isolate in sufficient quantities for biochemical assays and it can be quite challenging to image embedded osteocytes and their dendritic connections in three dimensions within intact bone specimens. Many of the current methods for imaging osteocytes, such as traditional ground sections, procion red staining, FITC staining and acid etched electron microscopy are actually imaging the lacunocanalicular network rather than the osteocytes themselves [14, 18,19]. With the explosion of interest in the field of osteocyte biology, there is an increased need for reliable ways to image these cells in live and fixed bone specimens.

Kamioka and colleagues developed methods for 3D confocal imaging of osteocytes using phalloidin staining of the actin cytoskeleton [20,21]. Building on this work, here we report the development of reproducible methods for 2D and 3D visualization of osteocytes in situ using multiplexed imaging approaches in which the osteocyte cell membrane, nucleus, cytoskeleton, and extracellular matrix can be imaged simultaneously in various combinations. We also present a new transgenic mouse line expressing a membrane targeted-GFP variant selectively in osteocytes as a novel tool for in situ imaging of osteocytes and their dendrites in fixed or living bone specimens. These methods have also been multiplexed with a novel method for labeling of the lacunocanalicular network using fixable dextran, which enables aspects of the osteocyte cell structure and lacunocanalicular system to be simultaneously imaged. The application of these comprehensive approaches for imaging of osteocytes in situ should advance research into osteocyte biology and function in health and disease.

Materials and methods

Preparation of fixed bone specimens

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee at the University of Missouri, Kansas City and conformed to relevant federal guidelines. The UMKC animal facility is operated as a specific pathogen free facility and is AAALAC approved. Animal care and husbandry conform to the Guide for the Care and use of Laboratory Animals (8th Edition), National Research Council.

For experiments on whole mount calvaria, the calvaria were harvested from humanely euthanized 5–7 day-old mice and washed with phosphate buffered saline (PBS). The mice were either (i) wild-type C57BL/6 mice; (ii) transgenic mice with osteocyte-targeted expression of a membrane targeted GFP (Dmp1-memGFP mice); or (iii) transgenic mice expressing a GFP-collagen fusion protein (GFP-collagen mice) (see below for a description of these transgenic mice). For widefield epifluorescence imaging of osteocytes in whole calvaria, the periosteum was carefully removed under a dissection microscope and surface osteoblasts were gently removed using a rubber policeman. For confocal microscopy, it is not necessary to strip the periosteum or remove osteoblasts, since the osteocytes can be imaged through the osteoblast layer. The specimens were fixed in cold 4% paraformaldehyde in PBS overnight at 4 °C with gentle rocking. Fixed half calvaria were then whole mount stained by immunofluorescent staining or using combinations of other fluorescent stains as outlined below, either with or without prior decalcification in 14% ethylenediaminetetraacetic acid (EDTA) pH 7.2 for 3 days.

For imaging of osteocytes in long bone specimens, femurs from C57BL/6 or GFP-collagen transgenic mice (ages specified in figure legends) were fixed for 48 h in 4% paraformaldehyde then decalcified in 14% EDTA. The decalcified bones were washed in PBS three times at 4 °C for 15 min with shaking and were then equilibrated at 4 °C in PBS containing 15% sucrose, followed by PBS/30% sucrose as a cryoprotectant. The bone specimens were embedded in OCT embedding medium (Tissue-Tek, PA, USA). Thick sections (50 or 100 µm) were cut using a Leica CM3050S cryomicrotome (Leica Microsystems, Wetzlar, Germany), and were collected into a 24-well plate containing PBS.

The thick sections were washed twice with PBS and were whole mount stained by immunofluorescent staining or using other fluorescent stains as outlined below.

Antibodies and reagents

Antibodies to the osteocyte membrane protein E11/gp38 [22] (also known as podoplanin) included a hamster monoclonal (gift from Dr. Andrew Farr, University of Washington, Seattle) and a polyclonal goat antibody against podoplanin (R&D systems, Pittsburgh, PA). Phalloidin conjugated to Alexa Fluor 488, Texas red or Alexa Fluor 633 was obtained from Molecular Probes/Invitrogen Corporation (Carlsbad, California) and was used to stain F-actin. The membrane dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), the nuclear stain 4'-6-Diamidino-2-phenylindole (DAPI) and a Texas red conjugated 10 kDa lysine-fixable dextran, used to image the lacunocanalicular system, were all obtained from Invitrogen Corporation. Detection antibodies and reagents used for immunostaining included Cy3 conjugated anti-hamster and anti-goat antibodies and a biotinylated anti-hamster antibody used in conjunction with FITC-streptavidin (Jackson ImmunoResearch, Westgrove, PA).

Generation of transgenic mice expressing GFP selectively in osteocytes

A transgenic mouse line expressing the *topaz* variant of green fluorescent protein (GFP_{tpz}) under control of an 8 kb fragment of the dentin matrix protein-1 (*Dmp1*) promoter has been previously described by Kalajzic and colleagues [23]. These mice express a GFP that is localized within the cytoplasm and this mouse line has been proven very useful for imaging/lineage tracing of osteocytes in vitro and in vivo [23,24] as well as for the generation of immortalized cell lines that recapitulate osteocyte differentiation [25]. However, in order to more clearly image living and fixed osteocytes and better resolve their dendrites in situ within bone, we have generated a new transgenic mouse line expressing a membrane targeted GFP variant (AcGFP1-mem) in osteocytes using the 9.6 kb fragment of the dentin matrix protein-1 (*Dmp1*) promoter to drive expression. The pAcGFP1-mem vector was obtained from Living Colors/Clontech Laboratories, Inc. (Mountainview, CA). The AcGFP-mem cDNA was retrieved from the pAcGFP1-Mem plasmid by XmaI and NotI restriction endonucleases, and was blunted at the NotI end. It was then subcloned into the XmaI and blunted XbaI sites of the pGL3-basic vector (Promega Corporation, Madison, WI) to replace the cDNA encoding firefly luciferase. The resultant construct was designated pGL-AcGFP1-Mem. The 14 kb *Dmp1* regulatory sequence, containing a 9.6 kb fragment of the *Dmp1* promoter region together with exon 1, intron 1 and the noncoding region of exon 2, was released from pSK vector by KpNI and XmaI (vector kindly provided by Dr. Jerry Feng, Texas A&M University Baylor College of Dentistry). This promoter has been previously shown to be highly expressed in osteocytes [26]. The promoter fragment was subcloned into the KpNI and XmaI sites of the pGL-AcGFP1-Mem plasmid to generate a 9.6 kb *Dmp1* promoter AcGFP1-Mem construct. This construct was designated pDmp1-AcGFP1-Mem. The transgene was released by SalI restriction endonuclease, separated from the vector backbone by agarose gel electrophoresis, and purified using Elutip-D columns (Whatman Schleicher & Schuell Bioscience, Inc. Keene, NH). Transgenic mice were generated on a C57BL/6N genetic background by pronuclear injection at the Transgenic Technology Center at the University of Texas Southwestern Medical Center, Dallas, TX. Founder mice were

identified by PCR of tail DNA samples using the following primers: forward primer, 5'-CCAAGCCCTG AAAATCACAGA-3', located on the *Dmp1* intron 1; and reverse primer, 5'-TCGCCGCTCACGCTGAACTT-3', located on AcGFP1-Mem cDNA. AcGFP1-Mem protein expression was confirmed by examining tail clip biopsies under the fluorescence microscope. Four founder mice showing strong AcGFP1-Mem expression in osteocytes were obtained and one of these lines (designated Dmp1-memGFP) that maintained high GFP expression was used for the present studies.

Transgenic mice with fluorescently tagged GFP-collagen

In addition to the Dmp1-memGFP transgenic mouse, we have also developed a transgenic mouse line expressing a GFP^{topaz} tagged collagen construct [27]. These transgenic mice were generated on a C57BL/6N background by the Transgenic Technology Center, University of Texas Southwestern Medical Center, as described above. The GFP^{topaz} tag was inserted into the mouse pro α 2(I) collagen N-terminus and expressed under control of the 3.6 kb type I collagen promoter. GFP-positive collagen is expressed and incorporated into the collagen fibers of the bone, skin, tendon, ligament, tooth, cornea, and other connective tissues. Thick frozen bone sections from these mice were also used for multiplexed imaging to enable us to simultaneously image the bone matrix surrounding the osteocytes together with other aspects of the osteocyte structure, such as their cytoskeleton, nucleus, and lacunocanalicular system.

Whole mount staining of bone specimens

Half calvaria or thick cryosections from the mouse femurs were blocked in PBS/1% normal donkey serum or PBS/1% normal donkey serum/1% bovine serum albumin (BSA) in a 48 well plate overnight at 4 °C. The samples were immunostained in primary antibody diluted in blocking buffer. Controls consisted of equivalent concentrations of species matched normal IgG (Jackson Immunoresearch, PA, USA). Samples were incubated in the primary antibodies for 2 h at room temperature or overnight at 4 °C, washed 5× with PBS and then incubated overnight at 4 °C with appropriate fluorescent detection antibodies (as stated in the figure legends) diluted in blocking buffer. To stain for F-actin, the sections were incubated overnight at 4 °C with Alexa Fluor 488-, Texas Red- or Alexa Fluor 633-conjugated phalloidin at 165 nM in blocking buffer. Two different methods were used for staining of the cell membrane with the lipophilic carbocyanine dye, DiI. One method involved diluting the DiI to 100 μ M in 100% ethanol and incubating overnight. The other method involved incubating the samples for 1 week in a solution of 100 μ M DiI in 50% DMSO:50% PBS. For nuclear staining of whole mount calvaria and thick bone specimens, the DAPI stain was used (4 μ g/ml in PBS) for 30 min at room temperature. To fluorescently stain for mineral in undecalcified specimens, Alizarin red complexone was used (50 μ g/ml in PBS) with overnight incubation at 4 °C with shaking. After these staining procedures, the sections or whole mount calvaria were washed 5× with PBS, mounted under a number 1.5 coverslip in 50% glycerol in PBS + 1 mM MgCl₂ and sealed using toluene/formaldehyde-free nail polish. In some experiments for confocal imaging with oil immersion, the samples were mounted in TDE (2,2'-thiodiethanol) mounting buffer. The TDE mounting was done by sequentially equilibrating the samples in increasing concentrations of TDE (Sigma-Aldrich, St. Louis MO) diluted in phosphate buffered saline (PBS). The procedure included

successive 2 hour incubations in 10% TDE, 25% TDE and 50% TDE followed by an overnight incubation in 97% TDE. Slices were then mounted on glass slides in 97% TDE: 3% PBS and sealed under a number 1.5 glass coverslip.

Imaging of lacunocanalicular system using lysine-fixable dextran

The osteocyte lacunocanalicular system can be readily imaged using injection of dyes such as procion red into the animal before sacrifice or using FITC staining of ground sections of bone [14,18,19]. However, these dyes are not fixable and are not retained after decalcification so they can only be used on undecalcified samples, which is not ideal for combining with other stains, such as immunostaining and staining of the cytoskeleton. We have therefore developed a novel method for lacunocanalicular imaging using a lysine-fixable dextran (Invitrogen Corporation). Mice were injected intravenously with a Texas Red labeled lysine-fixable 10 kDa dextran (32 mg/kg in PBS) and sacrificed 4–5 min following the injection. The femurs were fixed for 48 h in 4% paraformaldehyde in PBS. The samples were then decalcified and thick (50–100 μ m) sections were cut on the cryostat as described above.

Multiplexed imaging of osteocytes

The various stains described above for the osteocyte cytoskeleton, cell membrane, nucleus, lacunocanalicular system, and extracellular matrix/mineral were combined in various permutations for multiplexed confocal imaging. In some experiments these stains were combined with immunostaining for E11/gp38 or with using sections from the Dmp1-memGFP transgenic mouse to visualize the cell membrane or the GFP-collagen transgenic mouse to image the extracellular matrix. This approach allows us to image several aspects of osteocyte structure simultaneously, such as the nucleus, cytoskeleton, cell membrane, lacunocanalicular space, and extracellular matrix.

Microscopy and image processing

Specimens were viewed using one of the following microscopes, as indicated in the figure legends: (1) a Nikon E800 widefield epifluorescence microscope (Nikon Instruments Inc., Mellville, NY) configured with an Optronics CCD color camera (Optronics, Goleta, CA) and interfaced with the AnalySIS software (Soft Imaging System GmbH, Muenster, Germany); (2) a Zeiss LSM 710 scanning confocal microscope interfaced with the Zen 2011 software (Carl Zeiss Microimaging LLC, Thornwood, NY); and (3) a Leica TCS Sp5 II confocal microscope interfaced with the LAS AF software (Leica Microsystems, Wetzlar, Germany). Confocal image stacks were collected using compensation if required to correct for loss of signal intensity that occurs with increased depth penetration into the sample.

ImageJ software (v1.48v: Wayne Rasband, National Institutes of Health) was used for image processing applications, including color merging, Z projection, 3D projection, stack processing and generating movie .avi files. Software used for 3D rendering and/or deconvolution and image processing included the ImageJ plugin 3D viewer (Benjamin Schmid), LAS-AF software version 3.0.0 for TCS SP8 and Autodeblur & Autovisualize software (Media Cybernetics, Bethesda, MD).

Quantitation of signal intensity for comparisons of mounting agents

To compare the performance of mounting agents, phalloidin stained bone slices ($n = 3$ per group) were mounted either in glycerol:PBS mountant (50% glycerol: 50% PBS:1 mM MgCl_2) or TDE mountant (97% TDE, 3% PBS). Slices were imaged using the Leica TCS Sp5 II confocal microscope using identical confocal settings (same laser intensity and detector gain such that none of the samples saturated the detector during image acquisition) and without Z-plane compensation so that the drop off in signal intensity with penetration into the tissue could be measured. Image stacks were analyzed using ImageJ to determine the mean intensity of pixels containing fluorescent signal (i.e. pixels with a value of zero intensity were not included in the average). To monitor the drop off in signal with imaging depth, the average signal intensity was normalized to the intensity in the first Z plane and plotted against increasing Z plane number. Statistical analysis of the data was performed using a Bland–Altman test with a Student's t-test to determine if the bias between the two datasets was statistically significant from zero ($p < 0.05$).

Results

Imaging of intact osteocytes in whole mount preparations and thick bone sections

For investigators without access to confocal imaging systems, intact osteocytes can be readily imaged with good resolution in whole mount neonatal mouse calvaria using a widefield fluorescence microscope system if the periosteum are first stripped away to reveal the underlying osteocytes. Fig. 1 shows a whole mount neonatal mouse calvarium double stained for the osteocyte marker, E11/gp38, a membrane protein that in bone is specific for osteocytes, and phalloidin, which labels the actin cytoskeleton. In the inset image at the right note that the cell membrane (red) extends slightly beyond the boundary of the actin cytoskeleton (green) and note that on the tips of the dendrites, the membrane extends out beyond the limit of the actin cytoskeleton (small inset).

Confocal microscopy provides better resolution and is amenable to 3D imaging. Using double staining for phalloidin and DAPI, which stains the nucleus, high resolution confocal stacks were obtained from thick decalcified sections of adult mouse femur. Z projected images from these image stacks reveal the extensive interconnected dendrite network (Figs. 2A and B). Rendered 3D images of the same stacks reveal the intricate osteocyte dendrite organization in three dimensions (Fig. 2C and D) and illustrate the close interrelationship between osteocyte dendrites and vasculature within the bone cortex (Fig. 2C, arrowheads). Please see online Supplemental Movie 1 for an animated movie depicting the 3D osteocyte network.

Multiplexed imaging of the osteocyte cell membrane and development of a novel Dmp1-memGFP transgenic mouse

For imaging of the osteocyte cell membrane, we have developed a new transgenic mouse model in which expression of a membrane localized version of GFP (AcGFP1-mem) is targeted to osteocytes using the 9.6 kb Dmp1 promoter. Fig. 3A shows fluorescence images of tail vertebrae from a 2 week old Dmp1-memGFP transgenic mouse compared to a wild type littermate control. Note the strong green fluorescent signal in the bone shaft of the tail

vertebrae of the Dmp1-memGFP transgenic mouse and the lack of signal in the intervertebral disc or cartilage. The wild-type littermate shows no GFP signal. Within the tail vertebral bone, the GFP gives a punctate staining pattern, corresponding to its localization in osteocytes. Figs. 3B – E illustrate how this mouse can be used for multiplexed imaging of the osteocyte membrane (Fig. 3B) together with its cytoskeleton and nucleus (Fig. 3C). The merged image in Fig. 3D shows a multiplexed 3-color image of the osteocyte membrane, nucleus and cytoskeleton. The inset in Fig. 3D shows punctate GFP-positive vesicle-like structures which were observed throughout the bone matrix surrounding the osteocytes and in between dendrites. These vesicle-like structures do not stain with phalloidin and were an unexpected finding, suggesting that the osteocytes may shed vesicles into the bone ECM while they are embedding. Fig. 3E shows a 3D-rendered multiplexed image of the same field. See online Supplemental Movie 2 for an animation of 3D renders of Dmp1-memGFP and 3 color multiplexed imaging.

Since it is not always convenient for researchers to use the Dmp1-memGFP transgenic mouse for imaging of the osteocyte cell membrane, we also developed approaches for staining the osteocyte cell membrane using the lipophilic carbocyanine dye, DiI, and determined its suitability for multiplexing. This dye is often used as a neuronal tracer which can diffuse along the membrane of an individual neuron and into other cells that are directly connected to it [28]. We found that DiI staining gives different results in bone specimens depending on whether the staining is performed in 100% ethanol compared to 50% DMSO: 50% PBS. Fig. 4A shows staining with DiI in a 100% ethanol staining buffer in thick sections from a 5-month old mouse femur. The DiI gives nice staining of the osteocyte cell bodies and dendrites throughout the sample and also stains abundant vesicle-like structures in the bone ECM (see higher power image in Fig. 4B), similar to those seen in the Dmp1-memGFP transgenic mice. However, when DiI staining was performed in an ethanol buffer, we found that it was incompatible for multiplexing with phalloidin, as ethanol treatment disrupted the phalloidin staining, resulting in a diffuse staining pattern that does not reflect the correct localization of the actin cytoskeleton (data not shown). When the DiI staining was performed in a buffer of 50% DMSO:50% PBS, it gave a very different staining pattern in which some but not all osteocytes took up the lipophilic dye (see Fig. 4D). This is likely because in this more aqueous solution, the hydrophobic DiI cannot penetrate the tissue except via diffusion along cell membranes. Therefore, only osteocytes that are connected via their membranes to a surface osteocyte or other cell on the tissue surface can take up the dye — i.e. the dye is acting like a neuronal tracer. Interestingly, this technique also did not label the vesicle-like structures in the ECM. An advantage of performing the DiI staining in 50% DMSO:50% PBS is that it is compatible for multiplexing with phalloidin and DAPI, enabling simultaneous imaging of the osteocyte membrane, cytoskeleton and nucleus (see Figs. 4C – E and F).

Multiplexed imaging of osteocytes and their lacunocanalicular network

We next wanted to develop methods for imaging the osteocyte lacunocanalicular network simultaneously with various other aspects of the osteocyte. To accomplish this we have developed a novel approach for imaging the osteocyte lacunocanalicular system by injecting fixable fluorescently tagged dextran into the circulation prior to sacrifice. Because the

dextran is fixable it does not diffuse away during decalcification and can be multiplexed with various other fluorescent probes that require decalcification of the samples. Figs. 5A–E show multiplexed imaging of fixable Texas Red-labeled dextran together with phalloidin and DAPI, which enables simultaneous imaging of the lacunocanalicular system, the osteocyte and its dendrites as well as its nuclei. In the merged 3 color images in Figs. 5D, E and enlarged inset F, note that the fixable dextran stain gives a “halo” around the outside of the osteocyte cell body representing the lacunar space and that the dendrites can be seen to traverse across the lacunar space. This approach allows simultaneous 3D reconstructions of the osteocytes themselves together with the lacunocanalicular network. See online Supplemental Movie 3 for a 3D movie depicting the osteocytes and their lacunocanalicular network. Fig. 5G shows a more detailed 3D construction of an individual osteocyte revealing the level of detail that can be obtained using this approach (see online Supplemental Movie 4 for an animated 3D movie of this individual osteocyte)

Multiplexed imaging of osteocytes and their extracellular matrix

We have recently developed a new transgenic mouse model expressing a GFP-collagen fusion protein [27], which provides a novel approach for multiplexed imaging of various aspects of the osteocyte simultaneously with its extracellular matrix. Confocal imaging of GFP-collagen in decalcified thick sections from these transgenic mice reveals a considerable amount of substructure in the bone matrix (Fig. 6A). An unexpected observation was that many osteocyte lacunae have “rings” of bright and dark GFP-collagen fluorescence around them (Fig. 6A and inset), which most likely represent cycles of addition and removal of collagen in the perilacunar area by the osteocytes. Figs. 6B and C show 4-color multiplexed imaging of bones from GFP-collagen mice in which we have simultaneously visualized the collagen matrix using GFP-collagen, the osteocyte lacunocanalicular system using fixable Texas Red labeled dextran, the osteocyte cytoskeleton using Alexa Fluor 633 phalloidin and the cell nuclei using DAPI. Fig. 6B shows a Z-projected image and Fig. 6C shows a 3D rendered image. This can provide 3D information about the structure of the osteocytes, their lacunocanalicular network as well as the bone matrix simultaneously. In order to image the mineralized matrix together with 3D osteocyte structure, we have used alizarin red to stain the mineral component of the bone matrix in whole mount specimens or thick bone sections. Because of its fluorescence, this can be imaged by confocal microscopy and can be combined with staining of the osteocyte cellular structure using the approaches already described above. The example in Figs. 6D–F shows confocal imaging of a whole mount neonatal mouse calvarium stained with alizarin red combined with immunostaining for the early osteocyte marker, E11/gp38. The confocal imaging with alizarin red (Fig. 6D) reveals topographical features of the bone, such as vascular channels and osteocyte lacunae.

TDE mountant gives improved depth penetration and increased signal intensity for 3D osteocyte imaging

A recent study has reported that the mounting agent, 2,2'-thiodiethanol (TDE) is advantageous for high resolution confocal microscopy [29]. By equilibrating the samples in TDE mountant the refractive index of the tissue is matched to the immersion oil and it becomes possible to image deeper into tissue specimens without loss of signal. We therefore investigated whether TDE mountant had any benefit for 3D imaging of osteocytes in thick

bone slices. Fig. 7A shows comparative maximal Z projected images of phalloidin stained osteocytes from identical bone slices stained with phalloidin but mounted either in glycerol:PBS or TDE mountant and imaged under identical conditions on the confocal microscope. The TDE mountant resulted in a dramatic enhancement of signal brightness and allowed imaging deeper into the specimen. Fig. 7B shows quantitation of the change in phalloidin signal intensity plotted against Z-plane number (values for each plot are normalized to the signal intensity in the first Z plane). The glycerol:PBS mounting media showed a fairly rapid drop off in signal intensity over the first 30 Z-planes, followed by a more gradual loss of signal intensity over planes 31–300. By the 300th Z-plane (approximately 40 μm tissue depth) the signal intensity was reduced to about 35% of its original intensity. In contrast the signal intensity using TDE mountant remained stable for the first 30 Z-planes and then showed a more gradual loss over planes 31–300. By the 300th Z-plane, the signal intensity was still maintained at about 75% of its starting intensity. The Bland–Altman test/Student's t-test confirmed that the bias between the two datasets was significantly different from zero, confirming that the plots for glycerol:PBS vs. TDE mountant were significantly different from each other. Fig. 7C shows quantitation of the mean fluorescence intensity for TDE mountant compared to glycerol:PBS for Z plane #1, Z plane #30 (approx. 4 μm tissue depth) and Z-plane #300 (approx. 40 μm tissue depth). This shows that not only was the rate of signal drop off reduced with TDE mountant, but the starting signal intensity was also higher and the TDE mountant showed a significantly higher fluorescence intensity at all three tissue depths.

Discussion

With increased interest in the osteocyte as a key regulatory cell in the skeleton that controls osteoblast and osteoclast function, as well as an endocrine cell that regulates phosphate homeostasis, there has been a need to develop better approaches for imaging osteocytes in situ within skeletal tissues. Many of the available approaches for optical imaging of osteocytes have actually involved imaging of the lacunocanalicular system rather than the osteocytes themselves. This has been done by dye uptake approaches such as in vivo injection of procion red [14,19] or preparing ground sections of bone with or without FITC staining [18], or bodian staining [30,31]. Building on the pioneering work of Kamioka and colleagues, who developed approaches for 3D imaging of osteocytes in situ using fluorescent staining of the osteocyte actin cytoskeleton [20,21], we now present approaches for comprehensive 2D, 3D and multiplexed optical imaging of intact osteocytes in situ within skeletal tissues. These methods require the use of intact neonatal bones or thick 50–100 μm frozen sections of adult bones. The methods include imaging of the osteocyte cell membrane, cytoskeleton, nucleus, lacunocanalicular system and the bone extracellular matrix, which can be multiplexed in various combinations to obtain two, three and four color images of several aspects of osteocyte structure simultaneously. These approaches could also be multiplexed with immunofluorescent staining for any protein of interest.

Confocal microscopy is the modality of choice for imaging these thick bone specimens as it allows for high resolution optical sectioning and 3D reconstruction of intact osteocytes and the osteocyte network. Multiphoton microscopy is also an excellent choice as it would allow even greater depth of imaging. However, since some investigators do not have access to

these technologies we have also developed methods for imaging intact osteocytes in situ within neonatal mouse calvaria that can be successfully used with a traditional widefield epifluorescence imaging system. The key is to first strip off the periosteum and remove the cells on the bone surface using a rubber policeman. This procedure reveals the underlying osteocytes and allows the investigator to obtain clear 2D images of intact osteocytes and their dendritic processes.

To expand the toolkit for imaging of osteocytes in situ, we report here the generation of a novel transgenic mouse that expresses a membrane targeted GFP variant (AcGFP1-mem) under control of a 9.6 kb fragment of the *Dmp1*-promoter. The expression of the *Dmp1*-memGFP transgene is predominantly in osteocytes and also in odontoblasts (data not shown), similar to the *Dmp1*-GFP reporter line developed by Kalajzic and co-workers in which the *GFP^{topaz}* variant, which is a cytoplasmic variant of GFP, was used [23,32]. However the membrane targeted GFP variant (AcGFP1-mem) used in the present study facilitates high resolution imaging of the osteocyte plasma membrane and dendritic processes. Bone specimens from the *Dmp1*-memGFP transgenic mouse can be fixed and the membrane-targeted GFP can be used for multiplexed imaging of the osteocyte membrane in combination with the other fluorescent stains already described that reveal the cytoskeleton, nucleus or other aspects of osteocyte structure. Our new transgenic model can also be used for imaging of live osteocytes in situ within intact bones. It should therefore be a powerful new tool for live imaging of osteocyte membrane dynamics and the motile properties of osteocyte dendrites as well as the dynamic events that occur during the transition from osteoblast to osteocyte (manuscript in preparation).

An unexpected finding from examining the bones of *Dmp1*-memGFP transgenic mice was that there are small vesicle-like structures released from osteocytes that are incorporated into their surrounding matrix, presumably during the process of osteocyte embedding. Similar membrane-bound vesicle-like structures were observed in DiI stained adult femurs as well as whole mount calvaria immunostained for the membrane-bound osteocyte selective protein, E11/gp38 (data not shown). These vesicle-like structures have therefore been observed using three independent staining approaches that are all targeted at the cell membrane, lipid or membrane bound proteins. They were not observed with other stains such as phalloidin that do not target membrane associated structures. One possibility is that they simply represent a mechanism by which the osteocyte reduces its cytoplasmic volume during embedding into the bone matrix, since the cytoplasmic volume of an osteocyte is known to be reduced by up to 70% during maturation [33]. A second possibility is that these vesicle-like structures are extracellular vesicles, which are membrane bound particles released by many cell types that have been shown to carry a cargo of proteins, signaling molecules, mRNAs and microRNAs. These extracellular vesicles are thought to provide a mechanism for intercellular communication in many cell types (reviewed in [34,35]) and have recently been reported in osteoblasts [36]. The diameter of the osteocyte derived extracellular vesicles that we have observed is about 0.5–2 μm , which is consistent with the size of microvesicles, and studies are currently underway to determine their composition and function.

Since it is not always convenient or possible to use a membrane targeted GFP transgenic mouse for imaging of the osteocyte cell membrane, we developed other approaches for labeling the cell membrane, such as DiI staining, which could in theory be used on any standard bone sample, including samples from human bone biopsies. DiI is a fluorescent lipid dye that diffuses within cell membranes and is relatively insoluble in aqueous solution. In the neurobiology field, DiI is often used as a neuronal tracer which will diffuse along the membrane of an individual neuron and into other cells that are directly connected to it [28]. Interestingly, when thick bone sections were incubated in a DiI staining solution made up in 50% DMSO:50% PBS, we found that not all osteocytes throughout the entire (100 μ m) bone section were stained, but instead only a specific subset of osteocytes was labeled. The most likely explanation is that these osteocytes were connected to cells near the surface of the specimen that transferred the dye to internal osteocytes via membrane diffusion of the dye. Therefore, when the DiI staining method is performed in a staining buffer of 50% DMSO:50% PBS, this approach could potentially be used to assess osteocyte inter-connectivity. Notably, with this method that relies on membrane transfer of the dye, there was no staining of vesicle-like structures in the bone matrix, suggesting that they are discrete structures (i.e. not directly connected to the osteocytes) and therefore could not be reached by the dye via membrane diffusion from a surface osteocyte. In contrast, when DiI staining was performed in an organic solution of 100% ethanol, there was complete penetration of the tissue with the dye and all the osteocytes and their dendrites as well as the microvesicle-like structures in the bone ECM were stained. Therefore, depending on whether DiI staining is performed in ethanol or in PBS:DMSO solution, different information can be obtained. A drawback of using DiI in an ethanol staining buffer is that this is incompatible with phalloidin staining. We found that regardless of whether the phalloidin staining was performed in aqueous solution prior to the DiI staining or was done after the DiI staining following rehydration of the tissue to PBS, the ethanol treatment disrupted the phalloidin staining so that it gave a diffuse localization pattern that did not accurately represent the actin distribution (data not shown). This is most likely because ethanol destroys the phalloidin binding site on actin [37]. Ethanol buffers are also incompatible with GFP, as ethanol destroys the GFP fluorescence [38].

In this study, we present a novel approach for imaging the osteocyte lacunocanalicular system using injection into the circulation of a fixable fluorescent dextran dye. This dye has lysine residues incorporated into the dextran conjugate which allows it to be fixed using aldehyde fixatives. To our knowledge this has allowed for the first time the ability to simultaneously image the osteocyte lacunocanalicular system together with other aspects of osteocyte structure, such as the cytoskeleton, cell body and nuclei using confocal microscopy. Other published techniques, such as procion red labeling and FITC labeling also give excellent images of the lacunocanalicular system, but these approaches are not compatible with decalcification of the tissue, since this would lead to diffusion of the dye from the canalicular space. In contrast, the fixable dextran remains in place after decalcification and can subsequently be combined with immunostaining for antigens of interest or with fluorescent dyes that stain the cytoskeleton, nucleus, membrane, etc. This therefore allows for many possibilities in terms of multiplexed imaging to gain maximal information on the osteocyte and its lacunocanalicular system in the same specimen. The

disadvantage of the fixable dextran technique is that the canalicular staining is not as bright as with FITC or procion red labeling. Therefore, if multiplexing is not needed, the FITC or procion red staining methods would still be the methods of choice.

Previous studies have combined confocal imaging of the osteocyte lacunocanalicular system with imaging of bone mineral by synchrotron small angle X-ray scattering [39]. These studies analyzed the nanoscopic bone mineral particle size and arrangement in relation to the cell network and showed that most of the mineral particles in bone matrix are within 1 μm of the nearest osteocyte lacuna or canalculus. Here we present two new multiplexed confocal imaging approaches for imaging of the osteocyte together with its surrounding extracellular matrix that can expand the toolkit for these types of analyses. One approach uses undecalcified whole mount calvaria that are incubated overnight with alizarin red, a dye that binds to calcium in the bone ECM and then fluoresces red. This alizarin red staining of the mineral can be multiplexed with the other fluorescent staining approaches described above to obtain information on the osteocyte as well as the bone ECM in the same specimen. The second approach uses a GFP-collagen transgenic mouse developed in our laboratory in which the bone collagen is fluorescently tagged with GFP topaz . By multiplexing GFP-collagen imaging with fixable dextran, phalloidin and DAPI, images of the osteocyte matrix, lacunocanalicular space, cytoskeleton and nucleus can be generated. Both the GFP-collagen transgenic mouse and Dmp1-memGFP transgenic mouse can readily be multiplexed with most of the other fluorescent staining approaches we have described above in any desired combination. However, limitations include that fixation or treatment of the bone specimens with ethanol should be avoided as the GFP signal is dramatically reduced after exposure to organic solvents [38] and phalloidin staining is not compatible with ethanol. Additionally, care should be taken to avoid exposure to acidic or basic conditions as this may also result in dramatic loss of GFP signal [40]. An unexpected observation from imaging the bones of GFP-collagen transgenic mice was that several of the osteocytes have bright rings of GFP fluorescence around them, while others do not. This suggests that the osteocytes have synthesized new ECM in their perilacunar matrix and also suggests heterogeneity of osteocyte populations since some but not all the osteocytes have GFP-collagen perilacunar rings. These observations support recent reports in the literature that have re-examined the phenomenon of osteocyte remodeling of their perilacunar matrix [16,41]. It has been shown that osteocytes remove mineral from their perilacunar bone ECM during lactation and they then replace this mineral following weaning [16]. Our data with the GFP-collagen transgenic mice support the idea that the osteocytes can also add and remove the organic matrix in their perilacunar region.

In developing and optimizing the above methodologies for multiplexed imaging of osteocytes in bone tissues, we also investigated the use of a recently described mounting agent, 2,2'-thiodiethanol (TDE) that has been reported to be advantageous for high resolution optical microscopy [29]. When using high numerical aperture lenses for microscopy, refractive index mismatch between the immersion system and the medium in which the sample is mounted can lead to loss of image brightness and resolution, particularly when imaging deeper into the specimen (>10 μm). TDE is a non-toxic glycol derivative that is miscible with water. By adjusting the ratio of TDE to water, the refractive

index of the mountant can be precisely matched to the refractive index of the lens immersion oil. If the tissue samples are then equilibrated in this matched TDE mountant, the refractive index of the tissue is essentially matched to that of the immersion oil and it becomes possible to image much deeper into the tissue specimens without loss of signal. The TDE mountant has been reported to enhance the quantum yields of many fluorescent dyes compared to a PBS buffer [29]. In our experience using bone specimens, the TDE mountant performed much better than glycerol:PBS in terms of signal brightness and signal retention. The TDE mountant therefore resulted in improved resolution and enabled us to image much deeper into the bone tissue without loss of signal, while maintaining a lower laser power. This has clear advantages for generating confocal image stacks for 3D reconstructions of osteocyte networks and would be particularly advantageous for fluorophores that are sensitive to bleaching. However, in our experience, while the TDE mounting medium works well with stains such as GFP, DiI, DAPI and immunofluorescent staining, there are drawbacks that need to be considered when using it. Firstly, the procedure for TDE mounting takes an extra day as the samples have to be equilibrated in a graded series of increasing concentrations of TDE in PBS. Secondly, we found the TDE mounting medium to be less useful for imaging of phalloidin unless the confocal imaging could be completed within 1–2 days of sample mounting because with extended storage times of more than a few days, the phalloidin signal became diffuse and no longer accurately reflected its true localization within the cell. This is likely due to destabilization of the phalloidin conjugated fluorophores by the TDE [29]. It is also important to note that the usefulness of TDE mountant is limited to imaging with oil immersion lenses and there is no benefit when imaging with lenses that do not require oil immersion. Although the TDE mountant was clearly superior, it should be noted that fairly good results can still be obtained with mountants like glycerol:PBS if appropriate compensation settings are used when acquiring the images to correct for the loss of signal brightness with increasing tissue depth.

In summary, we have developed improved methods and new transgenic tools for 2D, 3D and live imaging of osteocytes, their lacunocanalicular system and their surrounding matrix that should facilitate the study of this important cell type in bone. These imaging approaches can be used to better understand the relationship of the osteocyte cell membrane, cytoskeleton, lacunocanalicular system and extracellular matrix as well as localization of specific proteins in various disease states, physiological states such as pregnancy and mechanical loading as well as in gain and loss of function transgenic mouse models and models of skeletal aging [42].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Widefield fluorescence imaging of intact osteocytes in a whole mount neonatal mouse calvarium. Immunostaining for E11/gp38 (cy3 anti-hamster antibody [red]) was combined with Alexa Fluor 488-phalloidin staining for F-actin [green]. Samples had the periosteum stripped off to reveal underlying osteocytes and were imaged on a Nikon E800 widefield fluorescence microscope. Inset images at the right reveal an individual osteocyte and its dendrites at higher magnification. Inset image at left shows the IgG control for E11/gp38 immunostaining. Bar = 10 μ m.

Fig. 2.
Confocal 3D imaging of phalloidin/DAPI stained osteocytes in adult mouse long bone. Thick (50 μm) frozen decalcified sections from a 4 week old mouse femur were stained with Alexa Fluor 488-phalloidin [green] and DAPI [blue]. (A) Maximal Z-projected confocal image (247 planes with 0.1 μm Z plane separation, 100 $\times$ oil lens, 1.7 $\times$ digital zoom with compensation). Note the intricate and highly organized dendritic network with an intracortical blood vessel visible at the left of the image; (B) enlarged inset from (A); (C) 3D rendered image of confocal stack from (A) showing 3D organization of osteocytes and

dendrites. Note the close association of two osteocytes and their dendrites with the intracortical blood vessel (arrowheads) (*also see* online Supplementary Movie 1); (D) enlarged inset from (C) showing 3D rendering of two osteocytes [imaging — Leica TCS Sp5 II confocal microscope; image processing — ImageJ; 3D volume rendering – 3D viewer in ImageJ]. Bar = 20 μm (A and C), 10 μm (B and D).

Fig. 3.
Dmp1-memGFP transgenic mouse for multiplexed imaging of osteocyte membrane, cytoskeleton and nucleus. (A) Low power fluorescence (upper panels) and DIC images (lower panels) of tail vertebrae from a 2 week old Dmp1-memGFP transgenic mouse (Dmp1-memGFP) compared to a wild-type littermate (WT). Note the specific localization of the GFP signal in bone. (B–D) Maximal Z-projected confocal images of osteocytes in a whole mount calvarium from a 7 day old Dmp1-memGFP transgenic mouse (40 planes with 0.13 μm Z plane separation, 100 $\times$ oil lens, 1.7 $\times$ digital zoom). (B) Osteocyte membrane

imaged using Dmp1-memGFP [green]; (C) osteocyte cytoskeleton and nucleus imaged using Texas Red phalloidin [red] and DAPI [blue]; (D) three color merge from B and C. The enlarged inset in (D) shows punctate GFP-positive vesicle-like structures in the bone matrix between osteocytes (arrowheads). (E) 3D rendered view of the same field (80 planes with 0.13 μm Z plane separation) (*also see* online Supplementary Movie 2) [imaging – Leica TCS Sp5 II confocal microscope; image processing – ImageJ; 3D rendering – 3D viewer in ImageJ]. Bar = 20 μm (B–D), 10 μm (E). 12.

Fig. 4. Multiplexed imaging of osteocyte membrane using lipid dye. Thick (50 μm) frozen decalcified femur sections were prepared from a 5 month old mouse. (A) Maximal Z-projected confocal images from sections double stained with DiI in ethanol staining buffer [red] and DAPI[blue] to image the cell membrane and nucleus (50 planes with 0.13 μm Z plane separation, 100× oil lens, 1.7× digital zoom). (B) Enlarged grayscale image of an individual osteocyte stained with DiI in ethanol buffer showing abundant punctate lipid containing vesicle-like structures throughout the matrix around the osteocyte (maximal Z

projection, 50 planes with 0.13 μm Z plane separation, 100 $\times$ oil lens, 1.7 $\times$ digital zoom). (C–F) Three color multiplexed imaging of osteocyte cell membrane, cytoskeleton and nucleus using (C) Alexa Fluor 488-phalloidin [green] and DAPI [blue] combined with (D) DiI staining [red] using a 50% DMSO:50% PBS staining buffer (maximal Z projection, 40 planes with 0.13 μm Z plane separation, 100 $\times$ oil lens, 1.7 $\times$ digital zoom). (E) Merged three color image of (C) and (D). (F) Enlarged image of two osteocytes (maximal Z projection of 10 planes) [imaging – Leica TCS Sp5 II confocal microscope; image processing – ImageJ]. Bar = 20 μm (A, C–E), 10 μm (B, F).

Fig. 5. Multiplexed imaging of osteocytes and their lacunocanicular space using combined staining with phalloidin, DAPI and fixable dextran – top row (A–C) maximal Z-projected confocal images of (A) Alexa Fluor 488-phalloidin [green], (B) fixable Texas Red-dextran [red] and (C) DAPI [blue] in a 50 μm thick femur section from a 10 week old mouse (50 planes with 0.13 μm Z plane separation, 100× oil lens, 1× digital zoom). (D) Maximal Z projected image of three color merge from A–C. (E) 3D rendered view of the same field (242 planes 0.13 μm Z plane separation). Note that the labeling of the lacunae with fixable

dextran (red) extends out beyond the limits of the osteocyte cell body and that the dendrites can be seen to traverse across the lacunar space. In several places the canaliculi can be observed to extend further than the dendrites. (F) Enlarged inset from (E). (G) 3D render of an individual osteocyte (front and back views) (*also see online Supplementary Movies 3 and 4*) [imaging — Leica TCS Sp5II confocal microscope; image processing – ImageJ; 3D rendering – LAS-AF software version 3.0.0 for TCS SP8]. Bar = 20 μm (A–E), 10 μm (F), 5 μm (G).

Fig. 6. Multiplexed imaging of osteocytes and their extracellular matrix – (A) Sum Z projected confocal image from a thick 50 µm frozen section of a 10 week old GFP-collagen mouse femur (10 planes with 0.13 µm Z plane separation, 100× oil lens, 1.7× digital zoom, with compensation). Note that the GFP-collagen reveals considerable substructure within the bone ECM and also note the rings of GFP fluorescence around osteocyte lacunae. Inset shows a high magnification view of an osteocyte lacuna with perilacunar GFP-collagen fluorescence. (B) Maximal Z projected 4-color confocal image from the same field of view

(100 planes with 0.13 μm Z plane separation, 100 $\times$ oil lens, 1.7 $\times$ digital zoom). The GFP-collagen is pseudocolored gray, actin cytoskeleton is shown with Alexa Fluor 633 phalloidin [pseudocolored green], lacunocanalicular system is shown with fixable Texas Red labeled dextran [red] and nuclei are stained with DAPI [blue]. (C) 3D rendered image of the same field in (B) (*see online Supplementary Movie 5 for animation of 3D rendered image*). (D) Maximal Z projected image of an undecalcified whole mount neonatal mouse calvarium stained with alizarin red and (E) merged image of same field showing alizarin red staining combined with immunostaining for E11/gp38 (biotinylated anti-hamster antibody/streptavidin FITC [green]) (74 planes with 0.43 μm Z plane separation, 40 $\times$ lens). (F) 3D isosurface rendered image of an enlarged area from (E) [imaging – Leica TCS Sp5 II (A–C) or Zeiss LSM 710 confocal microscope (D–F); image processing – ImageJ; 3D rendering – 3D viewer in ImageJ]. Bar = 20 μm .

Fig. 7.

TDE mountant increases signal intensity and imaging depth in bone specimens – (A) Comparison of TDE mountant and 50% glycerol:50% PBS mountant for confocal imaging of Alexa Fluor 488 phalloidin staining in thick decalcified sections from a 10 week old mouse femur (Maximal Z projection of 300 planes with 0.13 µm Z plane separation, 100× oil lens, 1.7× digital zoom, without compensation). Bar = 20 µm. (B) Quantitative comparison of TDE vs glycerol:PBS mountants plotted as fold change in mean signal intensity in relation to imaging depth (Z-plane number) [data for each Z plane are normalized to the first Z plane]. The Bland–Altman test/Student's t-test confirmed that the bias between the two datasets was significantly different from zero ($p < 0.05$). (C) Comparison of the mean fluorescence intensity of glycerol:PBS mountant compared to TDE at Z plane number 1, 30 (4 µm tissue depth) and 300 (40 µm tissue depth). Data are mean $\pm$ SD ($n = 3$). * = $p < 0.05$, ** = $p < 0.001$ compared to glycerol control (1 way ANOVA, followed by Tukey's post hoc test).