

Multiphoton microscopy for the study of osteocytes in bone

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INTRODUCTION: Osteocytes reside within the mineralized bone matrix in an extensive three-dimensional network of lacunae and canaliculi. This intricate microenvironment shapes osteocyte responses to mechanical stimulation, necessitating *in vivo* imaging to fully capture their physiological behavior¹. Two-photon (2P) microscopy has enabled direct visualization of osteocyte responses to mechanical load *in vivo*². However, light scattering and diffraction from the crystalline bone matrix limit 2P imaging depth and signal quality. Recently developed three-photon (3P) microscopy uses longer excitation wavelengths to achieve greater imaging depth and improved resolution compared to 2P. In the mouse brain, 3P imaging has reached depths exceeding 1 mm³, and similar approaches have achieved imaging through the cortical bone into the marrow space of the tibia⁴. Additional methods, such as refractive index (RI) matching adhesives and adaptive optics (AO), can further correct optical aberrations introduced by bone tissue⁵. Here, we quantitatively compare the image quality of 2P and 3P microscopy in mouse long bones and assess the improvements provided by diffraction correction strategies. We hypothesize that 3P imaging yields greater depth of signal preservation, normalized signal intensity, signal-to-noise ratio, and signal-to-background ratio compared to 2P, with further enhancement achieved using AO and RI-matching materials.

METHODS: Skeletally mature (16–18 week) female DMP1-Cre;GCaMP8m mice (JAX 037718) were used for proof-of-concept studies; future work will include males. The third metatarsal (MT3), tibia, femur, and humerus were dissected, fixed in zinc-buffered formalin, and soaked overnight in 1 mM CaCl₂ to enhance fluorescence. For adaptive optics (AO) correction in three-photon (3P) imaging, deformable mirror shape was manually optimized using Zernike polynomials⁶. For refractive index (RI) matching, Loctite 4305 adhesive (Henkel) and a 5 mm coverslip were applied and UV-cured⁵. Images were acquired at the mid-diaphysis through the dorsal or anterior surface. To assess signal decay, z-stacks were collected at constant laser power until fluorescence was lost (n = 2–4). Effective attenuation length (EAL) was calculated in MATLAB (MathWorks) as the depth at which the top 1% of signal intensity decreased by 1/e² (2P) or 1/e³ (3P). For image quality comparison, z-stacks were acquired with exponentially increasing power until cell signals were indistinguishable (n = 1/bone type). Cell regions of interest (ROIs) were generated in FIJI (NIH), and normalized intensity, signal-to-noise ratio (SNR), and signal-to-background ratio (SBR) were calculated. All procedures were IACUC-approved; significance was determined by two-way ANOVA with multiple comparisons (GraphPad Prism; p < 0.05).

RESULTS: Effective attenuation length (EAL) showed a general trend of reduced signal decay with three-photon (3P) compared to two-photon (2P) imaging, although a statistically significant difference was detected only between humeral 2P and 3P measurements (Figure 1). The addition of adaptive optics (AO) or refractive index (RI)-matching adhesive did not further improve EAL in 2P + glue or 3P + glue conditions. Image quality varied among bone types, as reflected by differences in cell brightness and contrast relative to background. In the third metatarsal (MT3), normalized cell intensity was greater in 2P than in 3P. The application of AO to 3P increased overall brightness to levels comparable to 2P; however, the addition of RI-glue equalized intensities across 2P, 3P, and 3P + AO. In the femur, the signal-to-background ratio (SBR) was higher in 3P than in 2P, particularly beyond 50 μm below the surface. AO further improved SBR within approximately 70 μm of the surface, and while RI-glue reduced the difference between 3P and 3P + AO, both remained greater than 2P. In the tibia, the signal-to-noise ratio (SNR) was modestly higher in 3P compared to 2P, with this trend persisting under AO correction. Unexpectedly, when RI-glue was applied, 2P images appeared substantially brighter than 3P or 3P + AO up to 80 μm below the surface (Figure 2a). Representative images acquired 70 μm below the surface highlight these brightness differences (Figure 2b). In the humerus, differences were only observed after RI-glue application, with normalized intensity in 3P + AO markedly lower than in 2P + glue or 3P + glue.

DISCUSSION: Multiphoton imaging performance in mouse long bone is bone specific. While three-photon (3P) microscopy generally improved normalized intensity, SNR, and SBR compared to two-photon (2P), these gains varied by site, with the tibia and femur showing the most pronounced benefits. Minimal differences in signal decay suggest that effective attenuation length (EAL) alone does not capture overall image quality. Instead, surface curvature and optical distortion strongly influence imaging outcomes, with narrower diaphyses responding best to adaptive correction. These results establish 3P microscopy as a powerful approach for deep osteocyte imaging in mineralized tissue while reaffirming the utility of 2P for select bone regions where its performance remains comparable.

SIGNIFICANCE: This study defines the practical capabilities of multiphoton microscopy in mouse long bones. By comparing two-photon (2P) and three-photon (3P) imaging performance, we identify both the depth limitations where 2P remains effective and the extended imaging range achievable with 3P. These findings clarify when each modality is best applied and highlight the potential of 3P microscopy to enable high-quality deep imaging of osteocytes in their native environment, opening new opportunities to investigate processes such as osteocyte interactions with cell populations in the marrow space.

REFERENCES: 1) You+, *J Biomech* (2001); 2) Lewis+, *PNAS* (2017); 3) Horton+, *Nat Photonics* (2013); 4) Rakhymzhan+, *iScience* (2024); 5) Wang+, *Nat Methods* (2018); 6) Coelho+, *Proc SPIE* 8588 (2013)

IMAGES AND TABLES:

