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Measuring strain using digital image correlation of second harmonic generation images

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ABSTRACT

The micromechanical environment of bone is crucial to understanding both bone fracture and mechanobiological responses of osteocytes, yet few techniques exist that are capable of measuring strains on the micrometer scale. A method for measuring micrometer level strains has been developed based on digital image correlation (DIC) of second harmonic generation microscopy (SHGM) images. Bovine tibias milled into thin sections were imaged using SHGM under loads of 0 and 15 MPa. Strains were measured using DIC and compared to applied strain values. First and second principal strains decreased in magnitude as the analysis region area increased from 1750 μm^2 to 60,920 μm^2, converging to 1.23 ± 0.74 and -0.745 ± 0.9816 times the applied strain respectively. A representative sample histogram revealed regions of pure tensile and compressive strain, and that strains were highly heterogeneous ranging from 8410 to −8840 microstrain for an applied 2870 microstrain. Comparison with applied strain measures suggested that analysis sizes of 1750 μm^2 and greater were measuring strains on the tissue scale, and higher resolution is required for collagen fibrillar strains. Regions of low SHGM intensity ("dark" regions) were seen which are believed to be lacunar and perilacunar regions of low collagen density. However, no significant differences in strain magnitude were present in dark regions versus regions of high signal intensity. The proposed technique is effective for strains on the size order of bone microarchitecture, and would be useful for studies into the mechanical microenvironment during loading. The technique also has potential for in vivo studies in small animal models.

1. Introduction

Bone is a mechanically regulated tissue with distinct tissue hierarchies that contribute towards sensing and control of material integrity (Turner, 2008). Insight into bone mechanobiology began with the study of global bone mechanics, but research has expanded to the mechanics of smaller organizational bone hierarchies (Klein-Nulend et al., 2005). Bone extracellular matrix at the smallest level is comprised primarily of type I collagen fibrils reinforced by apatite mineral crystals (Rho et al., 1998). Fibrils are then organized into collagen fibers and fiber bundles, the primary constituent of lamellae and osteons. Of particular interest is the individual contribution of collagen to the mechanical response of bone at different size scales. At the micrometer level of collagen fibril networks, bone fracture mechanics have been shown to be dependent upon fibril orientation (Peterlik et al., 2006). Collagen fibrils also provide fracture toughness, absorbing and dissipating fracture energy (Fratzl et al., 2004). However, efforts to study strain at this size regime have been computational or performed on decalcified bone samples, neither of which are fully representative of the in vivo mechanical behavior of calcified fibrils. Here, we aim to present a new method of measuring bone strain in calcified bone specimens using digital image correlation and second harmonic generation microscopy.

Bone strain measurements have often been taken using digital image correlation (DIC) (Bay et al., 1999; Verhulp et al., 2004; Bay 1995). DIC is a non-contact optical measurement technique originally developed for experimental mechanics applications to determine two-dimensional strains along the surface of a sample (Bruck et al., 1989). Previous work involving DIC and bone has focused mainly on the periosteum, or milling bone to expose lacunae and osteons. However, machining samples to expose lacunae and other features may alter the response of microfeatures to applied loads. Therefore, it could be advantageous to image intact lacunae and microfeatures using a method that penetrates below sample surfaces. Common non-destructive techniques for obtaining bone microarchitecture images include computed tomography, radiography and magnetic resonance imaging (Lespessailles et al., 2006). However, these techniques have limited resolution or it may be unfeasible to apply

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loads during imaging. Therefore, previous methods must be expanded upon in order to allow further study of the bone mechanical environment.

An ideal candidate for non-destructive imaging for digital image correlation is second harmonic generation microscopy (SHGM). SHGM is a non-linear optical method with micrometer level resolution and high tissue penetration depth. Beneficially, collagen fibrils produce high contrast, high resolution images under second-harmonic generation microscopy (Cox et al., 2003; Williams et al., 2005). Second-harmonic generation microscopy therefore provides an opportunity to quantify the extracellular matrix strains of calcified bone on a micrometer scale. Additionally, both SHGM and DIC have been used separately to image and analyze both cortical and trabecular bones (Lo Celso et al., 2009; Bay et al., 1999; Lespessailles et al., 2006). Taken together, SHGM and DIC are ideal candidates for determining collagen fiber strains in calcified bone tissue. This study aims to validate the use of digital image correlation and second harmonic generation microscopy for the measurement of strain in calcified bone on the micrometer scale.

2. Methods

2.1. Specimen preparation

Dogbone shaped samples of bovine tibia were created for mechanical testing. Tibia ends were removed by bandsaw, allowing quartering of the shaft (Fig. 1A). After quartering, each section was ground into a flat plate ($60 \times 5 \times 20\ mm^3$) using a Buelher Ecomet 3 grinder with water jet to reduce heating damage (Fig. 1B). Each plate was cut into rectangular specimens ($60 \times 3 \times 0.5\ mm^3$) with a Buehler Isomet 5000 saw using water cooling and slow feed rate to prevent damage. The rectangular specimens were cut into a dogbone shape via routing with water cooling, removing excess sample outside of a dogbone shaped stainless steel mold. Finally, specimens were polished with 600 grit sandpaper and wrapped in saline soaked gauze until tested (Nicolella et al., 2001). Samples were primarily taken from bone quarters containing the medial and lateral tibial sections, in the middle length of bone directly between the distal and proximal ends.

In order to hold the bone in the aluminum mechanical grips in a manner which prevented slipping or cracking during tightening, both flat sides of each dogbone sample were bonded to a small acrylic sheet via dental composite (Fig. 1C). The samples were prepared for bonding by demineralizing the dogbone ends in 1 N HCl for 30 s. Next, 2-hydroxyethyl methacrylate (HEMA) was bound to the demineralized bone surface via 30 s ultraviolet (UV) activation utilizing the initiators camphorquinone and N-phenylglycine. These were subsequently bonded to an acrylic sheet with a UV polymerizable mix consisting of bis-2-hydroxypropyl methacrylate (bisGMA), triethylene glycol dimethacrylate (TEGDMA), polymethyl methacrylate (PMMA), and the activators 1% w/v camphorquinone and 1% w/w N-phenylglycine).

2.2. Sample loading and imaging

A loading machine capable of applying stress to samples under a Zeiss LSM 5 Multiphoton Confocal microscope objective was developed. The loading machine was computer controlled and allowed recording of both applied force and sample displacement (Fig. 2).

The loading machine utilized a stepper motor coupled with a linear drive to provide a 0.39 μm resolution. A 225 N load cell mounted to a custom machined bracket measured applied loads with approximately 0.1 N resolution. Specimens were placed in the loading device and imaged using a Zeiss LSM 5 Multiphoton Confocal Microscope operating in backscattering mode. Images were taken at 800 nm excitation wavelength with a Chroma D405/30 filter (Chroma Technology Corp, Bellows Falls, VT) and a water-immersion W Plan-Apochromat $20 \times /1.0$ objective (Carl Zeiss Microscopy LLC, Thornwood, NY). A low-pass dichroic was also used to reflect the SHG emission from the sample. Emitted light was detected using an NDD PMT detector at 12-bit resolution and a gain setting of 346. Loads of 0 and 15 MPa were applied at constant tension, and sample displacement was measured by the encoder in the stepper motor. Images were captured at a 2048×2048 pixel size in the XY plane, with one pixel corresponding to an area of $0.82 \times 0.82\ \mu m^2$. Images were also taken at 1.0 μm spacing along the Z-axis in order to ensure the capture of target features that moved out of plane during loading. Z-stacks of 50–100 images were obtained and it took half an hour to image each sample.

2.3. Digital image correlation

Image planes that were identical between the 0 MPa and 15 MPa loading intensities were chosen for analysis by two-dimensional DIC, making the analysis a measurement of the bone matrix strains caused by increasing load from 0 to 15 MPa. Pre-processing of the images was performed to increase the accuracy of the sub-pixel displacement fields. First, the intensity range of each slice was normalized to the image stack maximum and minimum values. Histogram equalization also had the added benefit of simplifying thresholding of collagen dark regions seen in the bovine bone. The sample images were then smoothed by a Gaussian filter with a $3 \times 3 \times 3$ kernel with a sigma radius of 1, with sigma being the standard deviation of the Gaussian smoothing function. Smoothing of the images was performed due to recommendations in the literature which indicated low-pass filters helped reduce errors caused by cubic interpolations functions used in digital image correlation (Schreier et al., 2000).

Four bovine bone samples were analyzed by DIC at the 0–15 MPa loading regime, chosen to mimic physiological load levels. In order to determine the optimal analysis region size, analyses were run with 50×50–300×300 pixel region sizes increasing by 50 pixel intervals (0.82 μm per pixel in both the X and Y directions). Each individual region analysis was performed in a montage of 10 pixel steps in the X and Y directions to obtain a spatial grid of displacement data (Fig. 3).

The size of the analysis grid measured 41×41 DIC iterations in the x- and y-axis. The displacement grid underwent corrections for rotations detected between the analysis and reference image and was smoothed by a median filter with a 3×3 kernel. An outlier filter was developed that filtered values outside of 3 standard deviations from values within a 5×5 neighborhood in order to prevent artificially high strains from rare cases where an iteration failed to converge. Finally, the displacement grid was bicubically interpolated across the analysis image size.

2.4. Strain calculation

Once displacement fields were obtained, the strain values were calculated by a two-dimensional Savitsky–Golay filter developed from a two-dimensional kernel in literature (Pan et al., 2007). In brief, the filter applies a least-squares fit of the displacement field to linear equations

$$u(x,y) = a_0 + a_1 x + a_2 y \quad (1)$$

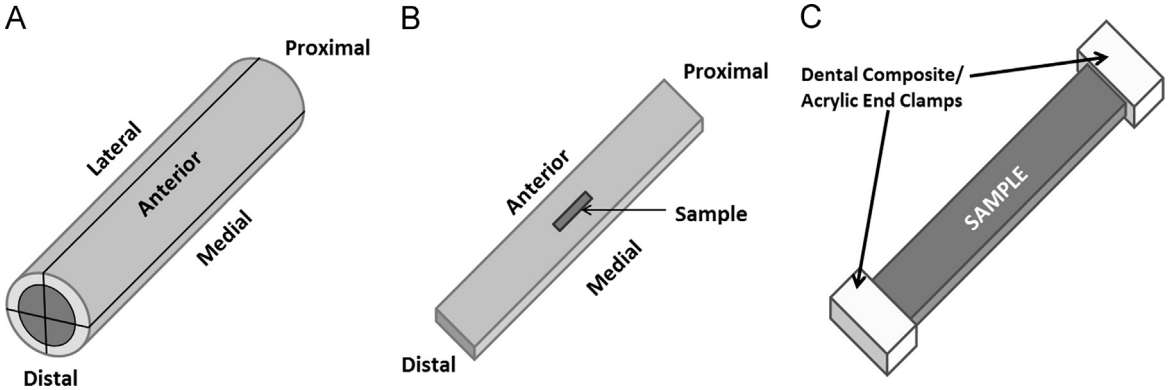

Fig. 1. Visual representation of sample preparation method. (A) After the tibia ends were removed by bandsaw, tibias were quartered along the long (distal–proximal) axis of the bone. (B) Tibia quarters were then ground into flat plates. Multiple bone sample strips were cut from each tibia quarter-plate. (C) Samples were routed into dog-bone shapes, and then the done bone ends were capped with a dental composite and acrylic sheets to enable clamping into the loading machine.