Modeling and Testing Dual-Colored Fluorescence Recovery After Photobleaching: A Novel Approach in Quantifying the Osteocytic Pericellular Matrix In Situ

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Introduction: Osteocytes are believed to sense load-induced fluid flow in the lacunar-canalicular system (LCS) through tethering pericellular matrix (PCM) fibers, which can be deformed under fluid flow like trees bent under wind [1, 2]. The PCM can also modulate the transport of signaling molecules and nutrients in bone [3, 4]. We hypothesize that the density of osteocyte PCM fibers regulates the sensitivity of osteocytes’ detection of and response to mechanical loading signals. Despite its physiological importance, quantitative study of the PCM in situ and in vivo is challenging, because the PCM is encased in the mineralized matrix, extremely thin and fragile, and susceptible to damages caused by routine histological processes. Our laboratory has recently developed a tracer velocimetry approach, combining fluorescence recovery after photobleaching (FRAP) and hydrodynamic modeling, to quantify osteocyte PCM in situ. However, in our previous studies using one-tracer FRAP approach [5], tracers that track fluid and solute velocities were injected and studied in separate mice, introducing data variability from both anatomical and loading inconsistency. The measurements of pericellular matrix were limited to mean values of a group of animals. In order to obtain pericellular matrix measurements for individual osteocyte lacunae and for individual animal, we propose a novel dual-colored FRAP approach, which allows simultaneous measurements of both fluid and solute velocities. We will first simulate the dual-colored FRAP in silico, and then test its feasibility in experiments. The long-term goal of our study is to elucidate the role of osteocyte PCM in bone mechanical sensing and signal transduction in vivo and in situ.

Methods: Dual-colored FRAP mathematical modeling: The simulation performed herein aims (i) to evaluate whether the ratio of the transport rates of the two tracers can be a surrogate indicator of the PCM fiber density and (ii) to determine how this outcome measure is affected by variations found in anatomical parameters. Similar to our previous studies [5], the region of interest is the osteocyte LCS 30 microns below the anterior-medial periosteal surface of a tibia. Using the three-compartment model developed previously [6], we examined the ratio of transport rates for two differently sized tracers (sodium fluorescein, radius 0.5nm; parvalbumin, radius 1.3nm) under 3N dynamic loading [5,6]. The PCM fiber volume fraction is parametrically varied from 0.5% to 50% while the anatomical parameters (lacunar size and spacing and canalicular number) are set to be 50%, 100%, or 200% of the mean values, which have been obtained in adult B6 tibiae using confocal and electron microscopy imaging [7]. The tracer diffusivities in each PCM configuration are estimated from their free diffusivities in water using Monte-Carlo simulations [8]. Load-induced flow velocities are obtained from a previously developed poroelasticity model [6]. The FRAP experiments are simulated with the three-compartment model (Fig. 1), where the central photobleached lacuna serves as transport sink and the surrounding lacunae serve as tracer reservoirs. The load-induced convection, in combination with diffusion, drives the tracers to move back and forth between the transport sink and reservoir sources. As tracers move through the PCM in the LCS, their transport rates are influenced by the density of the PCM due to the tracer-PCM interactions. The transport ratio of the large over small tracer will be examined for various LCS and PCM configurations, which mimic the in vivo anatomical variations. Dual-colored FRAP experiments: Sodium fluorescein (376Da, 10 mg/ml with 0.25 ml) and α-lactalbumin conjugated with sulfo-cy5 (14kDa, 10 mg/ml with 0.5 ml) were injected via the tail vein to one adult Balb/c female mouse. The tracers were allowed to circulate for 0.5 and 4h, respectively, prior to sacrifice. The tibiae were harvested, cleansed of soft tissue, and tested within 0.5-3h post-mortem. The experimental set-up consists of the Bose LM0 TestBench system integrated with a Zeiss LSM 510 confocal laser-scanning microscope [5]. A 40×0.8-numerical aperture water dipping lens attached to an objective inverter was used to capture images. A cluster of fluorescently labeled lacunae was identified and the central lacuna was subjected to FRAP tests [5]. In this preliminary study, tibia was held at static condition, while the time-series of fluorescence intensities of the two tracers were simultaneously collected in two channels (green for sodium fluorescein and red for lactalbumin) at a rate of ~1sec per frame and a frame-to-frame interval of ~6 sec, from which the transport rates of both tracers were obtained by performing linear regression on the logarithmic transformations of the normalized fluorescence recovery ratios of the photobleached lacuna (Fig. 2) [8].

Results: We found that the transport ratio of larger to smaller tracer monotonically decreased with increasing PCM fiber density (Fig. 3). The transport ratio was also affected by lacunar spacing and canalicular number, but was insensitive to the lacunar size.
In the dual-colored FRAP diffusion tests, the transport ratio of the large over small tracer was found to be $0.45 \pm 0.27$ for a total of 8 tests performed on 8 different lacunae.

**Discussion:** This study simulated and tested a novel dual-colored FRAP approach for quantifying the osteocyte PCM ultrastructure. The simulation results inform us that the transport ratio between the two tracers can be used as a surrogate measure of the PCM fiber volume fraction, because the two are approximately linearly related with each other (Fig. 3). The magnitude of the transport ratio is sensitive to the canalicular length and canalicular number, but not to the lacunar size, mainly because the tissue-level permeability is not related to the lacunar size [1,6]. Thus, in order to increase consistency of the pericellular matrix measurements, we need to carefully choose the target lacunae and the lacunae clusters for dual-colored FRAP experiments so that the canalicular length and canalicular number remain constant among tests. Because the canalicular number is proportional to the lacunar surface area [8], choosing similar sized lacunae can help maintaining the canalicular number to be consistent during the experiments. Furthermore, the diffusion transport ratio of larger to smaller tracer found in this study is comparable to our previous result using the one-tracer FRAP technique (a ratio of 0.53 for parvalbumin (12.3kDa) over sodium fluorescein [4]). This agreement supports the use of dual-colored FRAP approach in simultaneously and accurately measuring solute transport of two tracers, and for measuring osteocyte PCM measurement in loaded bones [5]. We are currently applying mechanical loading to murine tibia samples while using the dual-colored FRAP approach to measure changes in osteocyte PCM in bones of different age and of different mechanosensitivity (such as B6 vs. C3H) [10]. Our ultimate goal is to rigorously test the novel hypothesis that PCM fibers serve as osteocyte mechanosensors and thus regulate bone’s response to mechanical loading.

**Significance:** Osteoporosis is a growing health problem with increased risk of fractures. The dual-colored FRAP is a novel approach to quantify the osteocyte PCM, a potential target to regulate osteocytes’ mechanosensitivity for treating osteoporosis.

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**References:**

**Fig 1.** The three-compartment LCS transport model where tracers move with convection and diffusion between the central photobleached lacuna (sink) and the surrounding reservoirs (source compartments) during cyclic loading. A PCM fiber matrix is present between the cell process and the canalicular wall as shown in the blowup of the canaliculi (modified from Zhou, 2008).
Fig 2. A representative dual-colored FRAP experiment. (A) Both small sodium fluorescein (green) and large lactalbumin (red) penetrated into bone and stained the osteocyte lacuna. (B) Both tracers recovered the fluorescence intensity after photobleaching. (C) Large tracer recovered at a slower transport rate (shown in the smaller slope) than the small tracer.
Fig 3. The effects of A) lacunar spacing (d), B) canalicular number (N) and C) lacunar major axis dimension (a) on the transport ratio $k_{large}/k_{small}$ as a function of osteocyte PCM fiber volume fraction $k_{vf}$.