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# Dual photon excitation microscopy and image threshold segmentation in live cell imaging during compression testing

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ABSTRACT

Morphological studies of live connective tissue cells are imperative to helping understand cellular responses to mechanical stimuli. However, photobleaching is a constant problem to accurate and reliable live cell fluorescent imaging, and various image thresholding methods have been adopted to account for photobleaching effects. Previous studies showed that dual photon excitation (DPE) techniques are superior over conventional one photon excitation (OPE) confocal techniques in minimizing photobleaching. In this study, we investigated the effects of photobleaching resulting from OPE and DPE on morphology of in situ articular cartilage chondrocytes across repeat laser exposures. Additionally, we compared the effectiveness of three commonly-used image thresholding methods in accounting for photobleaching effects, with and without tissue loading through compression. In general, photobleaching leads to an apparent volume reduction for subsequent image scans. Performing seven consecutive scans of chondrocytes in unloaded cartilage, we found that the apparent cell volume loss caused by DPE microscopy is much smaller than that observed using OPE microscopy. Applying scan-specific image thresholds did not prevent the photobleaching-induced volume loss, and volume reductions were non-uniform over the seven repeat scans. During cartilage loading through compression, cell fluorescence increased and, depending on the thresholding method used, led to different volume changes. Therefore, different conclusions on cell volume changes may be drawn during tissue compression, depending on the image thresholding methods used. In conclusion, our findings confirm that photobleaching directly affects cell morphology measurements, and that DPE causes less photobleaching artifacts than OPE for uncompressed cells. When cells are compressed during tissue loading, a complicated interplay between photobleaching effects and compression-induced fluorescence increase may lead to interpretations in cell responses to mechanical stimuli that depend on the microscopic approach and the thresholding methods used and may result in contradictory interpretations.



## 1. Introduction

In biomechanics, it is often of interest to study the response of connective tissue cells to chemical or physical stimuli in order to understand the origin of a disease or the effectiveness of a treatment intervention (Jones et al., 2005). Changes in cell morphology can provide crucial clues of the effect of mechanical stimuli on tissue response (Bush and Hall, 2001; Guilak, 1994; Guilak et al., 1995; Han et al., 2009), and histology has been the conventional approach to address such questions in the past (Clark et al., 2003; Eggli et al., 1988). However, histological approaches have limitations, including cell shape distortion caused by tissue handling, dehydration of samples, or shrinkage of samples during fixation (Errington et al., 1997; Guilak, 1994; Jones et al., 2005). Most importantly, histological approaches only allow for assessment at one-time point and for static conditions, thereby ignoring the potentially more important dynamic mechanical and signaling responses of cells (Errington et al., 1997; Guilak, 1994; Jones et al., 2005).

The advent of fluorescence confocal laser scanning microscopy (CLSM) provided a non-invasive approach to study in situ cell mechanics dynamically (Guilak, 1994; Jones et al., 2005). In CLSM, live cells are stained with a fluorescent dye, the fluorophores are excited by laser light (one photon excitation (OPE)), and they emit fluorescent light for detection (Jones et al., 2005). The fluorescent signals are then collected in planar sections to generate a series of digital planar images, which, when combined, provide three-dimensional (3-D) live cell visualization. Since laser

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exposure excites all fluorescent agents along the light path, OPE microscopy uses a confocal pinhole to eliminate non-target areas (Inoué, 2006; Jones et al., 2005).

However, CLSM suffers from photobleaching artifacts that cannot be easily quantified or eliminated. Photobleaching leads to a decrease in fluorescence intensity and reduced image contrast. This affects the quantification of cell morphology and may lead to misinterpretations of cell responses.

Dual-photon excitation (DPE) microscopy was introduced about two decades ago and showed promise in minimizing photobleaching (Denk et al., 1990). In contrast to OPE microscopy, DPE microscopy excites the fluorescein molecules simultaneously with two photons of higher wavelength but lower energy. The highly localized excitation of DPE microscopy makes it attractive, because it significantly decreases the duration of fluorophore excitation, thus minimizing photobleaching.

The digital images of cells must be isolated from the surrounding extracellular matrix (ECM) for morphological measurement (Bush and Hall, 2001; Guilak, 1994; Guilak et al., 1995; Han et al., 2009). This is typically done with so-called threshold segmentation techniques (Sezgin and Sankur, 2004), in which a cut-off intensity (threshold) is chosen to divide the gray-levels into above the threshold comprising cells, and below the threshold comprising the ECM. Automatic threshold selection has been used to avoid user bias. Since thresholding methods isolate cells based on the current fluorescence intensity, they have been used to eliminate photobleaching effects (Abusara et al., 2011; Bush and Hall, 2001; Han et al., 2009). However, there is an abundance of automatic thresholding methods (Sezgin and Sankur, 2004), each based on different selection criteria, therefore, giving different results. Care must be taken to select the optimal threshold intensity, as too high a threshold will cause apparent cell shrinking, while too low a threshold introduces background noise into the cell environment (Ridler and Calvard, 1978). Therefore, it is imperative to assess the effectiveness of thresholding methods when quantifying cell morphology.

While previous studies provided evidence that DPE microscopy reduces photobleaching compared to OPE techniques (Bush et al., 2007), the aim of this study was to critically evaluate the effect of photobleaching of OPE and DPE microscopy on morphologies of connective tissue cells after repeat laser exposure. Experiments were performed using articular cartilage chondrocytes subjected to multiple laser exposures in loaded and unloaded conditions. Selected automatic thresholding methods were evaluated for their ability to reliably reproduce cell morphologies under conditions of no change and for unconfined compression of articular cartilage samples. We hypothesized that cell morphology changes associated with repeat DPE exposure are smaller than changes associated with repeat OPE exposure. Furthermore, we hypothesized that different automatic thresholding methods ('Otsu', '40% maximum intensity' and 'Ridler–Calvard') produce different cell morphology results. Finally, we hypothesized that loading of articular cartilage affects the fluorescence intensity of cells, thereby potentially masking the effects of photobleaching to an unknown extent.

## 2. Methodology

### 2.1. Cartilage explant preparation

Metatarsal-phalangeal joints of 24 month-old cows were obtained from the local abattoir. Osteochondral blocks were aseptically harvested from the medial load bearing surface of the joints and maintained in serum-free Dulbecco's Modified Eagle's Medium (DMEM, D5921) supplemented with 16 mM HEPES (H0887), 1.6 mM L-glutamine (G7513), 160 U/ml Penicillin–160 μg/ml Streptomycin (P4333) and 0.68 mM L-ascorbate (A5960) (Sigma Aldrich, Canada). Explants were cultured in a 5% $CO_2$-supplemented incubator at 37 °C until experimentation (within 2 days) (Changoor et al., 2010; Moo et al., 2011).

On the day of experimentation, a cylindrical, full-thickness cartilage sample attached to the underlying bone was cut using a circular punch. Cells in each specimen were positively stained at room temperature (21 °C) with calcein AM (8 μM; excitation$_{max}$: 488 nm; emission$_{max}$: 515 nm; Molecular Probe, OR, USA) and carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) (0.2 mM; excitation$_{max}$: 492 nm; emission$_{max}$: 517 nm; Molecular Probe, OR, USA) for 30 min. CFDA-SE was added to visualize the cartilage tissue to establish initial contact during compression testing. After staining, samples were washed three-times for 15 min in dye-free phosphate buffered saline (PBS) and then attached to a specimen holder using dental cement. The cartilage specimens were immersed in serum-free DMEM throughout the experiment to prevent dehydration (Guilak et al., 1995; Han et al., 2009).

### 2.2. Laser scanning microscopy

Cartilage samples were set-up in a custom-made compression system that allows for cell imaging during tissue compression (Han et al., 2009). An upright Zeiss Axio Imager.Z1 laser scanning microscope LSM510 NLO with a 40 × /0.8 N.A. and 0.17 mm coverglass-corrected water immersion objective (Zeiss Inc., Germany) was used for image acquisition. OPE was achieved using a 488 nm Argon laser (Zeiss Inc., Germany). The confocal pinhole size was set to 1 Airy unit. DPE was carried out using a mode-locked Chameleon XR infrared laser (Coherent Inc., USA) with 780 nm wavelength and the pinhole set to maximum. For both techniques, the laser intensity was adjusted to optimum levels to avoid pixel saturation. A low detector gain of 460 V was used to ensure good image contrast. A series of cell images (pixel size: 0.41 μm × 0.41 μm; bit-depth: 8; pixel dwell time: 2.56 μs; frame scan time: 1.57 s) were acquired along the objective axis ($z$-axis) at intervals of 1 μm using internal (de-scanned) detection.

### 2.3. Image processing

Only chondrocytes that maintained high contrast and staining over the entire experiment were selected for analysis. Every planar image in the image stacks was analyzed using the three thresholding methods: 'Otsu' (Otsu, 1979), 'Ridler–Calvard' (Ridler and Calvard, 1978) and '40% maximum intensity' (Bush and Hall, 2001). The highest threshold intensity was chosen for image segmentation. Three-dimensional (3D) reconstruction of chondrocytes was performed using custom-written code (VTK, the Visualized toolkit: Kitware Inc.) for cell volume computation (Alyassin, 1999). The density maps produced by the code were best-fit to an ellipsoid to quantify cell shapes (Feddema and Little, 1997). Cell widths and depths were defined along the major and minor axes of the cross section taken perpendicular to the cell height, respectively.

### 2.4. Calibration

Fluorescent latex microspheres of 6.0 μm diameter (excitation$_{max}$: 441 nm; emission$_{max}$: 486 nm; Polysciences Inc., PA, USA) were embedded in 2% agarose gel (Han et al., 2009). Image acquisition and processing are explained in Sections 2.2 and 2.3, respectively. Fifteen microspheres were selected for analysis. Image thresholds were calculated using the three methods mentioned in Section 2.3. The average apparent height of the microspheres in the $z$-axis was calculated. The correction factor for $z$-focus distortion was obtained for the three thresholding methods by dividing the known microsphere height by the average apparent height of the microspheres (Guilak, 1994; Han et al., 2009).

### 2.5. Photobleaching test

For the photobleaching tests, cartilage specimens were left uncompressed in the in situ compression system. Eight joints were randomly partitioned to the OPE or the DPE microscopy group. Cells ($n=20$ each for OPE and for DPE) located within 40 μm from the articular surface were studied. Seven consecutive laser scans without rest were performed using OPE and DPE.

Three analyses were performed on all image stacks. First, cell image threshold intensities were determined in the first scan and left constant for the remaining six scans to quantify the apparent cell volume decrease caused by photobleaching. Photobleaching-induced changes in the $x$-, $y$-, $z$-direction was quantified by the decrease in cell width, depth, and height, respectively. Second, image thresholds were determined for every scan (scan-specific thresholds) to offset the photobleaching effect. Changes in cell volume (mean ± 1SEM) over the seven scans were compared for the three thresholding methods. Third, we calculated the average decrease in cell volume caused by photobleaching for each scan and used these values as laser-exposure error factors. Cell morphologies were first determined using thresholds obtained in the first image scan and, based on the duration of laser exposure, error factors were summed with '1' to yield correction factors,