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Two-Photon Excitation Laser Scanning Microscopy of Human, Porcine, and Rabbit Nasal Septal Cartilage

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ABSTRACT

Two-photon excitation laser scanning microscopy (TPM) was used to image human, porcine, and rabbit nasal septal cartilage. TPM provides optical sections of thick tissue specimens *in situ* without the use of exogenous dyes or need for tissue fixation. The cartilage tissue was imaged using near-infrared light generated by a mode-locked titanium/sapphire laser that was raster-scanned and coupled to an inverted microscope. Absorption of two photons by endogenous molecules and subsequent fluorescence was filtered to specific spectral bandwidths and detected with photomultiplier tubes. Two-photon stimulated fluorescence was detected with photomultiplier tubes optimized to specific spectral bandwidths. Signal intensity corresponds to the concentration of fluorophores, principally NADH, NADPH, and flavoproteins hence providing a means of redox imaging the cellular metabolic state. Specimens were scanned from the surface to a depth of about 150 μm. Image size was 50 × 50 μm with a diffraction limited pixel size of 0.4 μm. Cell membranes, nuclei, and matrix structures were identified in human, pig, and rabbit tissues. TPM provides a means to study three dimensional chondrocyte structure and matrix organization *in situ* at substantial depths, and permits longitudinal examination of cultured tissue explants without the need for exogenous dyes, tissue preparation, or fixation.

INTRODUCTION

SPECIFYING the chondrocyte response to chemical and physical stimuli is the first step toward understanding the mechanisms behind cartilage tissue growth and regeneration. Biochemical studies show that cytokines, chemical reagents, or changes in physical environment alter cartilage matrix metabolism, but detailed analysis of the cell and tissue responses *in situ* are better obtained with microscopy. While the

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use of radionucleotides and immunohistochemical stains have allowed for functional imaging, these generally require fixation of tissue and cannot be used reliably to serially examine a specimen maintained in tissue culture over time. With conventional techniques, there is always a trade-off between the quality of functional versus structural information. As tissue engineering, biomechanics, and laser-mediated reshaping research progress, it will become increasingly necessary to observe changes in chondrocyte structure and function *in situ* over time without irreversibly damaging the specimen. The microenvironment of the chondrocyte is unique since the tissue consists primarily of a matrix of type II collagen, with aggregating proteoglycans and cells interspersed in this matrix either singly or in small groups. The structural relationship between the cellular and matrix components of the tissue is important in understanding how that biomechanical and biochemical function can be maintained.

Conventional confocal laser scanning microscopy (CLSM) has been used extensively to study living cartilage tissue and is a technique that enables the collection of detailed information about the *in situ* localization of cell and matrix molecules.[1–3] However, CLSM has significant limitations, which include (1) the use of exogenous stains which are diffusion limited in distribution; (2) shallow penetration depth due to the shorter wavelengths; (3) photobleaching due to the high-intensity laser source; and (4) toxicity to living tissue (photothermal and photochemical injury), which precludes high-resolution vital imaging. While the use of ultraviolet (UV) sources in CLSM enables excitation of endogenous fluorophores, these fluorophores suffer the limitations listed above. In addition, UV CLSM requires complicated optical designs that only partially compensate for inherent chromatic aberrations at UV wavelengths.[4,5]

Two-photon excitation laser scanning microscopy (TPM) is a technique that overcomes the limitations of conventional microscopy and provides for diffraction limited optical sections of thick tissue specimens *in situ* without the use of exogenous dyes or need for tissue fixation.[6–8] In cells, the major fluorophores are nicotinamide-adenine dinucleotide (NADH), reduced nicotinamide-adenine dinucleotide phosphate (NADPH), and flavoproteins (concentrated within the mitochondria); their fluorescence intensity (and hence concentration) is a direct measure of cellular respiration and provides a means of redox imaging the cellular metabolic state. Further, within the matrix, collagen will emit providing information on tissue structure.[9,10] These fluorophores are excited at about 360 nm and emit close to the 400–500 nm region. In two-photon microscopy, excitation results from the simultaneous absorption of two photons of twice the absorption wavelength of the UV fluorophore, causing a transition to an excited electronic state normally reached by higher energy UV stimulation. Two-photon excitation scanning confocal microscopy with near-infrared (NIR) excitation provides high fluorescence collection efficiency from endogenous fluorophores with reduced photodamage. Inasmuch as excitation is produced by the short pulse duration (~100 fs), high-intensity, and NIR sources, photothermal tissue injury and photobleaching are minimized, while tissues can be imaged at large depths of penetration (several hundred microns in many tissues). As less than a nanojoule is delivered to the tissue per pulse (at a typical average power of 10 mW and pulse repetition rate of ~80 MHz), one would not expect to observe optical breakdown.[11,12] This is substantiated by the observation that image signal intensity does not change despite repeated imaging for several minutes.

Two-photon excitation scanning confocal microscopy has been used to image several tissues and organs including the cornea,[13] skin,[14,15] reproductive organs,[16,17] inner ear hair cells,[18] and neuronal dendrites.[19] *In situ* imaging of physiological processes such as phagocytosis,[20,21] capillary blood flow in neocortex,[22] glucose metabolism,[23] and neuronal dendritic activity calcium[24] have also been studied using TPM. In this study, we illustrate the use of two-photon excitation laser scanning microscopy to study hyaline cartilage obtained from human, porcine, and rabbit nasal septa.

MATERIALS AND METHODS

Tissue specimens

Excess human nasal septal cartilage specimens were obtained from two patients undergoing routine nasal surgery at the University of California Irvine with the approval of the Human Subjects Institutional Review Board. Ten porcine and 10 rabbit septal cartilages were obtained from animals sacrificed for use in other protocols immediately postmortem in accord with Institutional Animal Care and Use Committee at UC

Irvine guidelines. The human and porcine specimens were cleaved in a sagittal plane with a custom guillotine to remove any remnant perichondrial tissue. The most superficial surfaces of the thin (<1 mm) rabbit septa were not removed; hence, a thin layer of perichondrium remained. Six-millimeter-diameter punch biopsies of the cartilage specimens were subsequently washed three times in calcium and magnesium free phosphate-buffered saline (PBS) with antibiotics (gentamicin [200 mg/L] and amphotericin B [22.4 mg/L]). Specimens were then placed in Dulbecco's modified Eagle's media containing gentamicin (50 μg/L), amphotericin B (5.6 μg/L), penicillin (100 U/mL), streptomycin (100 μg/mL), 10% fetal bovine serum, and 29.2 μg/L L-glutamine) and maintained at 37°C in a humidified 5% CO_2 atmosphere. All specimens were examined within 24 h of harvest. In each specimen, 15–25 fields (at 63×) were examined from superficial levels to a depth of 150 μm. Typically one to two chondrons were identified per high-power field (63×). A total of 10 porcine and rabbit septal cartilages and two human septal cartilages were imaged.

Two-photon excitation laser scanning microscopy

The cartilage tissue was imaged using a two-photon scanning microscope (Fig. 1). The TPM system consists of a 5-W Verdi laser (Coherent, Santa Clara, CA), which is used to pump a Mira 900F Titanium:Sapphire (Ti:Al_2O_3) laser (Coherent, Santa Clara, CA), which provides the two-photon pulsed excitation source. The ultrafast Ti:Sapphire laser is tunable between 690 and 1,000 nm, thus allowing for two-photon imaging of many UV excitable fluorophores. The wavelength used in this study was 780 nm. While this wavelength is not at the maxima of the excitation spectra of NAD(P)H (e.g., 720 nm [360 nm $\times$ 2]), it was selected to capitalize on the gain characteristics of the Ti:Sapphire source. The higher power (and longer wavelength) permits deeper optical penetration, particularly in turbid media such as cartilage.[25] Further, the two-photon excitation spectra of most flurophores are broader and flatter than their single photon counterparts.[8] The average power after the Ti:Sapphire laser is controlled using neutral density filters. Thus low average powers required for tissue and cell survival could be maintained at the sample surface (5–10 mW) while still maintaining sufficient peak power for two-photon excitation to occur. The mode-locked, 100-femtosecond, 76-MHz pulse train exiting the Ti:Sapphire laser is expanded, directed through a short pass dichroic beam splitter (700 nm, Chroma Technology, Brattleboro, VT) and collimated using two lenses to fill the back aperture of the microscope objective.

Tissue specimens were placed on a coverglass positioned in the center of an inverted microscope stage (Zeiss Axiovert 100 microscope; Zeiss, Thornwood, NY). A small drop of PBS was used to minimize refractive mismatches. The beam is scanned across the sample with a PC controlled X-Y scanner (series 603X; Cambridge Technology, Inc., Watertown, MA) using a custom interface and software. A Zeiss 63×, 1.2 N.A., water immersion objective having a working distance of 200 μm was used for these studies (Zeiss). The two-photon fluorescence from the tissue passes through a short pass dichroic beam splitter and is directed to a single photon counting detection system using a system of mirrors and lenses. The detection system consists of two photomultiplier tubes (PMTs; Hamamatsu Corp., Bridgewater, NJ) arranged perpendicularly (and separated by a long pass dichroic beamsplitter (580 nm, Chroma Technology), one optimized for green light (R7400P) and the other for red light (R7400P-01), thus allowing for simultaneous detection of fluorescence in two different wavelength regions. A lens and band pass filter focus the fluorescent signals onto the PMTs. Specimens were scanned from the surface to a depth of about 150 μm.

RESULTS

Figures 2, 3, and 4 are images of human, rabbit, and pig nasal septal cartilage, respectively, obtained using the TPM set-up described above. Chondrocytes in Figures 2 and 3 are likely isogenous. Each image was obtained at a depth of approximately 150 μm. Image size is 50 $\times$ 50 μm, with a pixel size of 0.4 μm[25]; this corresponds to a magnification of 63×. A false color look-up table was applied to signal intensities to aid with feature identification. Regions with high signal intensity (representing fluorophore concentration) are yellow and light blue. Low signal intensities are represented by darker colors.

Three chondrocytes are identified within a chondron in Figure 2. The cells are all roughly the same size, though the plane in which this image was obtained results in the observed size discrepancy among these

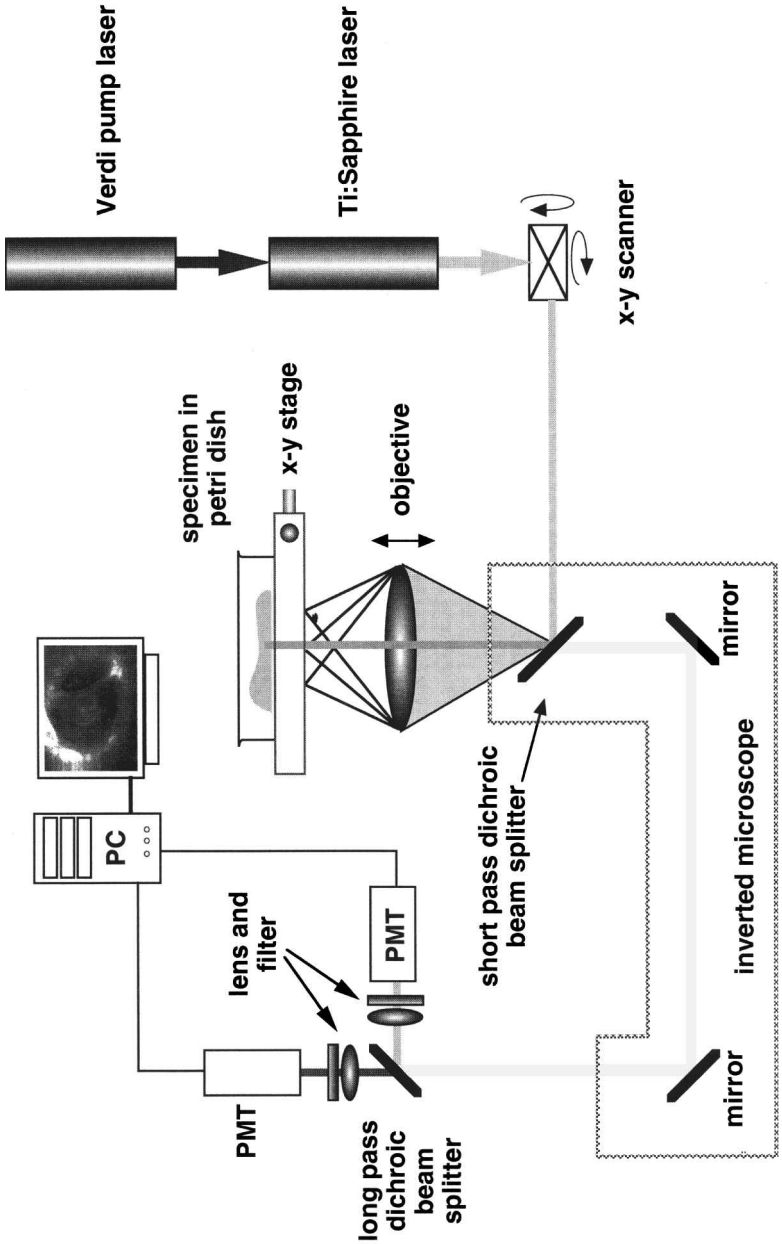

FIG. 1. Schematic of two-photon microscope.

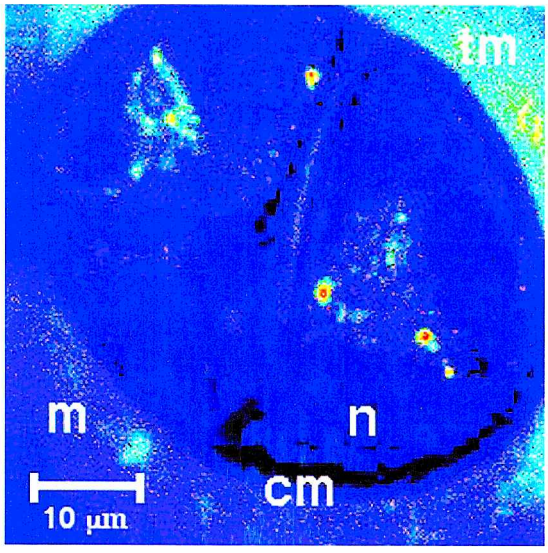

FIG. 2. Two-photon image of human septal cartilage at a depth of approximately 150 μm (tm, territorial matrix; m, matrix; cm, cell membrane; n, nucleus).

cells. High autofluorescence signals are observed in the matrix (m) surrounding the chondron. Signal intensity increases in the immediate vicinity of the cell membranes; this is likely due to changes in matrix protein density found in the territorial matrix (tm). In contrast, low-intensity signals are observed in the regions where the lipid-rich cell membranes (cm) reside, which form dark bands around each cell and the chondron as a whole. In the left-most chondrocyte, the image plane includes a large intracellular oval structure that is likely the nucleus (n). The cytoplasm of all three cells is heterogeneous with respect to signal intensity. As organelles and subcellular structures are below the diffraction limited resolution of the TPM, no other intracellular structures are clearly identified. The intermediate signal intensity of the cytoplasm, particular in regions of high signal intensity, may represent fluorescence from NADH/NADPH molecules

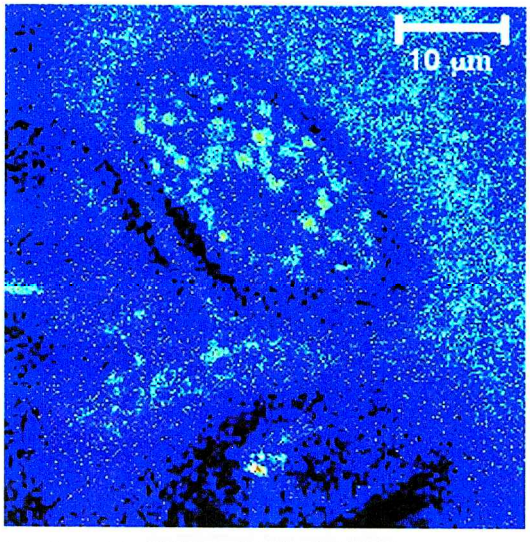

FIG. 3. Two-photon image of rabbit septal cartilage at a depth of approximately 150 μm.

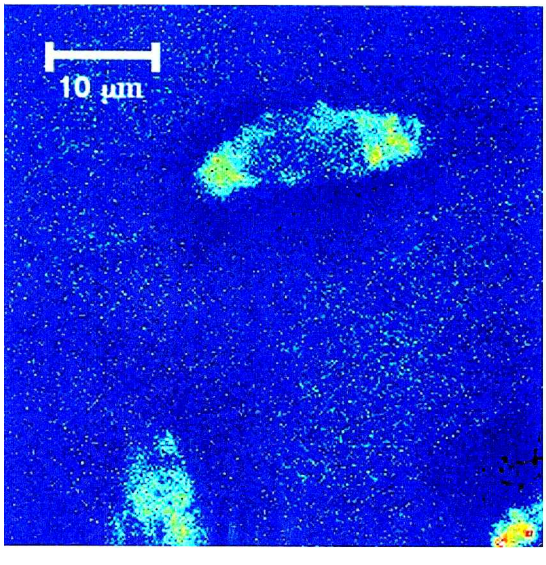

FIG. 4. Two-photon image of pig septal cartilage at a depth of approximately 150 μm.

within mitochondria. The three small isolated spots of extremely high signal intensity are likely due to detector saturation.

Rabbit septal tissue exhibits similar features (Fig. 3). Three individual chondrocytes in separate chondrons are identified. The individual chondrocytes are approximately the same size as their human counterparts, though with a more oval shape. The territorial matrix region, cell membrane, nucleus, and cytoplasm are once again identified. Distinct high-intensity signals are identified in the cytoplasm of the cell located in the upper left area of the image; these signals may represent a cluster of mitochondria within the cytoplasm. The shape differences of the three cells is likely due to the orientation of the cells within the tissue matrix and the plane of section of the image.

The chondrocytes identified in porcine septal tissue do not share the classic features observed in human and rabbit specimens. In general, the cells are elongated with less distinct demarcation between the cytoplasm and the matrix (Fig. 4). Within the cytoplasm, regions of low signal intensity are identified, albeit no structure suggesting a nuclear membrane is seen. The cells are also smaller than their human and lagomorph counterparts. Porcine septal cartilage is unusual in that it preferentially warps along the caudal-cephalic axis of the tissue once the overlying perichondrium is removed (unpublished results). This warping occurs within minutes and might explain the more spindle-like shape of the cell.

DISCUSSION

This study was designed to illustrate the utility of TPM in imaging chondrocytes in their native environment and providing survey information on chondrocyte features in nasal cartilage from three different species. The images illustrated in Figures 2–4 were selected to represent some of the consistent features we observed in this survey of three tissues. This study was not meant to provide a rigorous morphometric analysis of chondroctye geometry *in situ*, although this is a future objective of our research. In this study, we have compared images obtained from live *ex vivo* human, rabbit, and porcine nasal cartilage. We demonstrated that features of the chondrocytes and surrounding matrix may be imaged without interfering with their normal architecture. Imaging cartilage tissue with TPM would allow for longitudinal studies of living chondrocytes in situ without the need for exogenous dyes, tissue preparation, or fixation. As many biochemical and molecular studies of cartilage require enzymatic digestion of the cellular matrix, TPM has the advantage of permitting evaluation of both cellular and matrix components in their native state, and provide a means to study three-dimensional chondrocyte structure in the native matrix. Inasmuch as preservation of cellular phenotype is dependent upon the matrix architecture, *in situ* observation eliminates artifacts that would accompany dedifferentiation that occurs in isolated specimens.

The imaging of nasal cartilage has immediate application for the development of techniques to reshape and remodel facial cartilage but the data also indicates the potential for imaging hyaline cartilage from other sources such as intervertebral discs and articular cartilage, where the matrix components are essentially the same as nasal cartilage but the precise cell/matrix architecture is unique. Degenerative diseases affecting these tissues are a major area of investigation. Articular cartilage is routinely used in explant culture systems for biochemical studies which give little information about changes in organization of the matrix components. The application of TPI to these systems will allow for longitudinal studies where the effects of application of inflammatory mediators or growth factors known to change matrix synthesis and deposition can be studied in the same tissue sample. At most locations, articular cartilage is only a few hundred microns to several millimeters in depth from the articular surface to the bone cartilage interface and functional changes at different depths in the cartilage have been observed by differential dissection or immunocytochemical studies. Using our nasal cartilage specimens, we have successfully imaged to a depth of 150 μm. This technique has the potential to allow advances in our approaches to studying early morphological changes in degenerative conditions such as rheumatoid arthritis and osteoarthritis and understanding how these changes influence disease progression.

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