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# A Diagnostic System for Articular Cartilage Using Non-Destructive Pulsed Laser Irradiation

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**Background and Objectives:** Osteoarthritis involves dysfunction caused by cartilage degeneration, but objective evaluation methodologies based on the original function of the articular cartilage remain unavailable. Evaluations for osteoarthritis are mostly based simply on patient symptoms or the degree of joint space narrowing on X-ray images. Accurate measurement and quantitative evaluation of the mechanical characteristics of the cartilage is important, and the tissue properties of the original articular cartilage must be clarified to understand the pathological condition in detail and to correctly judge the efficacy of treatment. We have developed new methods to measure some essential properties of cartilage: a photoacoustic measurement method; and time-resolved fluorescence spectroscopy.

**Materials and Methods:** A nanosecond-pulsed laser, which is completely non-destructive, is focused onto the target cartilage and induces a photoacoustic wave that will propagate with attenuation and is affected by the viscoelasticity of the surrounding cartilage. We also investigated whether pulsed laser irradiation and the measurement of excited autofluorescence allow real-time, non-invasive evaluation of tissue characteristics.

**Results:** The decay time, during which the amplitude of the photoacoustic wave is reduced by a factor of $1/e$, represents the key numerical value used to characterize and evaluate the viscoelasticity and rheological behavior of the cartilage. Our findings show that time-resolved laser-induced autofluorescence spectroscopy (TR-LIFS) is useful for evaluating tissue-engineered cartilage.

**Conclusions:** Photoacoustic measurement and TR-LIFS, predicated on the interactions between optics and living organs, is a suitable methodology for diagnosis during arthroscopy, allowing quantitative and multidirectional evaluation of the original function of the cartilage based on a variety of parameters. Lasers Surg. Med. 43:421–432, 2011. © 2011 Wiley-Liss, Inc.

**Key words:** osteoarthritis; photoacoustic measurement; time-resolved autofluorescence spectroscopy; tissue-engineered cartilage



## INTRODUCTION

Osteoarthritis is thought to affect about 30 million people in Japan [1], but is not a direct threat to life. However, this condition both affects activities of daily living and diminishes quality of life among sufferers, so the associated human and social loss is difficult to estimate. The disease involves dysfunction caused by cartilage degeneration, but objective methodologies of evaluation based on the original function of the articular cartilage are currently unavailable. Evaluations that are currently used to establish conservative therapies or the prognosis of surgery as a treatment for osteoarthritis are merely based on patient symptoms or the degree of joint space narrowing on X-ray images. Accurate measurement and quantitative evaluation of the mechanical characteristics of cartilage (viscosity, elasticity, and lubrication) are important, and the tissue properties of the original articular cartilage need to be recognized if the pathological condition is to be understood in detail and treatment effects judged accurately. The development of such evaluation technologies is thus required to facilitate a functional diagnosis of osteoarthritis. If these evaluations can be achieved non-invasively, an accurate understanding of the pathologies should be possible, allowing the planning and performance of treatments for locomotor apparatus diseases that accompany the degeneration of cartilage, such as osteoarthritis. Such evaluations would also be useful as objective tools in situations such as the clinical

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trials of new drugs. A better understanding of the pathological condition in detail and determination of prognosis based on a body of clinical data should thus be possible. This will in turn facilitate the careful planning of treatments according to the specific pathological conditions of individual patients, improving activities of daily living, and enhancing the lives of many people.

Recent studies have suggested ultrasonography (US) as a sensitive method for determining cartilage thickness [2,3], structural properties [4], surface roughness [5], and enzymatically induced, specific degeneration of the superficial collagen network [6–8]. Mechanically, the collagen network is primarily responsible for the dynamic properties of cartilage by constraining transversal expansion, whereas proteoglycans contribute predominantly to interstitial fluid flow and the equilibrium response of cartilage [9,10]. Structural and mechanical properties vary within and between different articular surfaces [11,12]. Hattori et al. [13,14], reported a method to assess joint cartilage using US. By limiting examination to the mechanical properties of joint surfaces, cartilage can be assessed by indentation testing [15–17]. For the clinical diagnosis of mechanical properties, as with indentation testing, US requires arthroscopy. Quantitative intra-articular US imaging [18–20] and Optical coherence tomography [21,22] have been already been applied in vivo during knee surgery. MRI excels at geographical mapping, and while one advantage is the ability to gather a wide variety of extra-articular data, unlike US or indentation testing, viscoelastic properties cannot be directly measured. Normal and abnormal signals on images are simply compared to indirectly estimate mechanical properties. Several quantitative MRI techniques have recently been introduced for the non-invasive assessment of structural and mechanical properties of articular cartilage [23]. T2 mapping is sensitive to the integrity of collagen networks, collagen content, and fibril orientation [24–26]. T1 mapping in the presence of Gd-DTPA2 contrast agent, namely delayed gadolinium-enhanced MRI of cartilage (dGEMRIC), reflects the proteoglycan distribution in cartilage via the inverse distribution of ionic contrast agent [27,28].

The method of assessing cartilage function using a non-invasive pulsed laser that we have been analyzing is a technique focusing on interactions with the body when the laser is applied to cartilage. In other words, this photoacoustic method is a technique for measuring viscoelastic properties based on how sound waves travel and attenuate through the body and resembles US. Time-resolved laser-induced autofluorescence spectroscopy (TR-LIFS) measures autofluorescence generated through other interactions, and analyzes the properties of characteristically collagen-rich cartilage tissue matrix using the same laser irradiation. In other words, with our proposed method employing pulsed laser irradiation, interactions are measured by two different methods to obtain more biological information than US or indentation testing (Table 1). Relaxation times as measured by the photoacoustic method agreed well with the intrinsic viscoelastic parameters, with a correlation coefficient of 0.98, when

**TABLE 1. Comparison of Major Cartilage Measurement Devices**

| | Indenter | Ultrasound | Laser (LIPA + TR-LIFS) | MRI |
|---|---|---|---|---|
| Direct measurement of viscoelasticity properties | Possible (up to superficial layer) large impacts of force application and superficial layer | Possible (average from surface to deep layers) | Possible (average from surface to deep layers) | Unable |
| Assessment of surface structures (fibrillation, etc.) | Possible | Possible | Possible | Unable |
| Compostional information (collagen content) | Unable | Possible (imaging) | Possible (distinguishable COL1 and COL2) | Possible (imaging) |
| Arthroscopic environment | Necessary | Necessary | Necessary | Unnecessary |

LIPA, laser-induced photoacoustic measurement; TR-LIFS, time-resolved laser-induced autofluorescence spectroscopy; COL1, collagen type 1; COL2, collagen type 2. Viscoelasticity can be measured directly using an indenter, ultrasound system, or laser. However, these devices all require arthroscopy. Conversely, MRI excels at geographical mapping and is advantageous for gathering a wide variety of extra-articular information, but the mechanical properties of abnormal signals can only be estimated indirectly based strictly on image changes, and delineating fine joint surface structures is difficult. Using a laser, COL1 can be differentiated from COL2. This method is thus suited for assessing tissue properties.

tissue-engineered cartilage tissues cultured for various periods (up to 12 weeks) were used as samples. By comparing the results of biochemical analyses and biomechanical studies, we confirmed the photoacoustic signal as a good indicator for evaluating extracellular matrix formation in order to determine the characteristics of tissue-engineered cartilage [29–38]. We also developed a method for extracellular matrix characterization using TR-LIFS, which enabled simultaneous measurements with mechanical properties using the photoacoustic method [39–41].

We propose the application of these unique measurements and evaluation methodologies [29–41], which we have developed in vitro to non-invasively assess regenerating cartilage (tissue-engineered cartilage) and to diagnose cartilage degeneration. When used in clinical settings, our laser measurement method requires arthroscopy, but the amount of information obtained is greater than that provided by other devices (Table 1). This method may thus be a useful assessment technique in clinical studies that closely assess cartilage.

## MATERIALS AND METHODS

Scattering, reflection, and increase in temperature attributable to absorption and the production of fluorescence and acoustic waves are regarded as the main effects when light or laser beams irradiate living organs to be measured (Fig. 1) [42]. A non-invasive and selective diagnostic device that uses optics via a fiber optic cable has recently attracted attention. This device is based on a technology that takes advantage of interactions between optics and living organs. Use of these interactions enables simultaneous collection of not only morphological information, but also various physiological and biochemical data, so the potential for use as a diagnostic device is greater than that of techniques based on a single type of information, such as ultrasonic waves. Bioinstrumentation and imaging with a laser beam, which have recently attracted attention, show features that facilitate the application of this technology to medical fields. We have focused on the interactions between living organs and optics (particularly photoacoustic waves and fluorescence), measured a variety of parameters related to these interactions when induced by the same laser, and developed a system that allows the simultaneous evaluation of the mechanical characteristics and properties of tissue (Fig. 2) [32,33,38].

### Evaluation of Mechanical Characteristics Using a Photoacoustic Method

Tissue viscoelasticity affects the propagation and attenuation of the stress waves induced by pulsed laser irradiation [29]. The relaxation time of the stress wave, calculated as the time in which the amplitude of the stress wave decreases by a factor of $1/e$, gives the intrinsic relaxation parameters ($\eta/G$) of the tissue, where $\eta$ is the viscosity and $G$ is the elasticity. We have proposed a basic principle whereby the mechanical characteristics of the tissue can be measured using photoacoustic parameters. In this measurement technique, the relaxation time of the

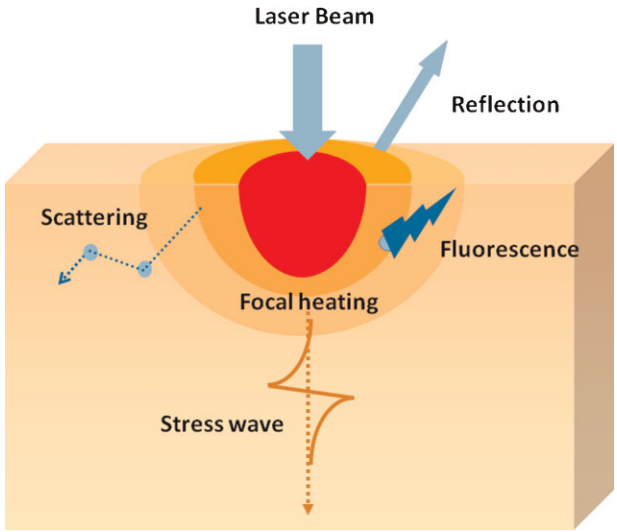



Fig. 1. Mutual interaction between light and a living body. The scattering, reflection, and increase in temperature attributable to absorption and the production of fluorescence and acoustic waves are regarded as the main effects when light or laser beams irradiate living organs to be measured. The figure was cited and modified from Bioengineering: Principles, Methodologies, and Applications ISBN: 978-1-60741-762-0 Authors: M. Sato et al., Editors: A. Garcia et al., pp. 179–190, 2010 Nova Science Publishers, Inc. Chapter 7.

stress that acts on a linear viscoelastic object (consisting of a spring and a dashpot) is related to the viscoelastic parameters of the object, and to the damping time of the stress waves generated by irradiation with a nanosecond pulse laser. Relaxation time is theoretically related to the viscoelastic ratio [42]. The relaxation time ($\tau$) is calculated using the Levenberg–Marquardt algorithm, a nonlinear least-squares method, as follows. When the stress wave intensity is attenuated only by its reflection at the boundaries and its relaxation during its transmission through viscoelastic materials, then the time course of the stress wave intensity is expressed by the following equation [34]:

$$I_\delta = I_0 \times R \times \exp\left(\frac{-t_\delta}{\tau}\right),$$

where $I_0$ is the intensity of the stress wave at $t = 0$, $R$ is the product of reflectivity (the product of the internal reflectivity at the interface at both ends of the sample), $t_\delta$ is the time after laser irradiation, and $\tau$ is the damping time of the stress wave and corresponds to the viscoelastic ratio.

As the optimum wavelength of the laser beam was unknown at the beginning of this study, we used an optical parametric oscillator (Spectra-Physics, Tokyo, Japan) with the original probe (Fig. 3) and set the oscillation wavelength within the range of 250–355 nm, with collagen and protein as the optical absorbers.

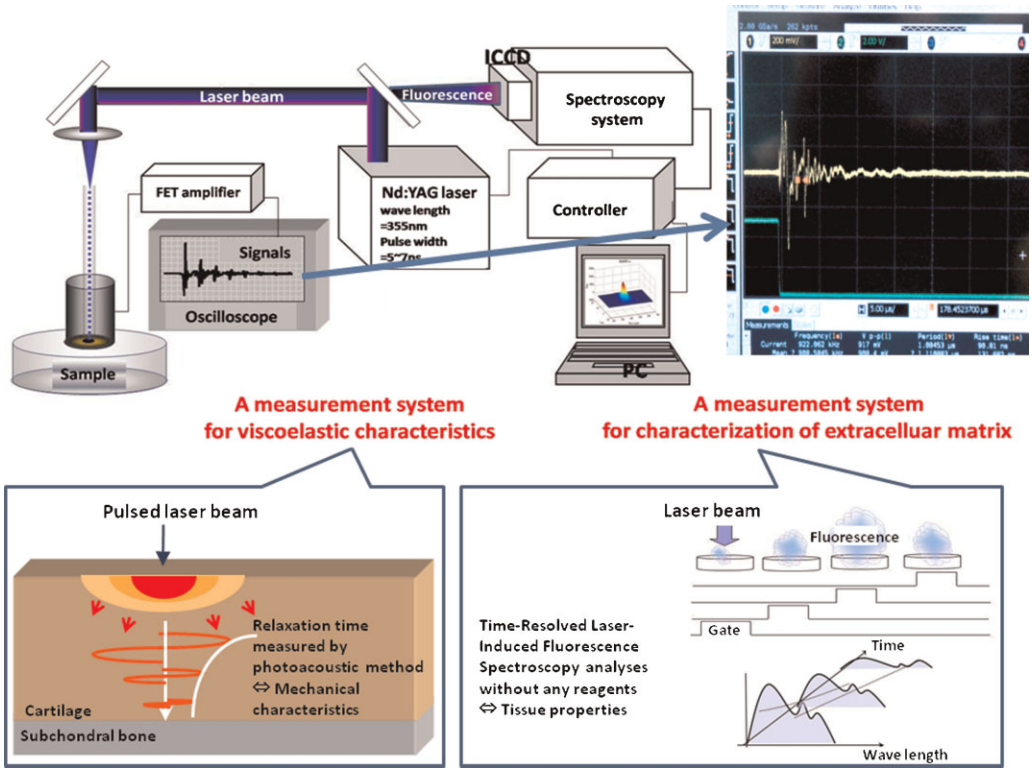



Fig. 2. Simultaneous measurement system: photoacoustic measurement of viscoelastic characteristics and fluorescent measurement with time-resolved autofluorescence spectroscopy. From a practical perspective, a commercially available 3rd (355 nm) harmonic Q-switched Nd:YAG laser (pulse width, 5–6 nanoseconds) was used for the excitation light source in the present study. The light beam was focused with a lens and then coupled to a silica fiber of 400 μm in core diameter. Transmitted light energy was maintained at approximately 50 μJ/pulse. Thermoelastic waves induced by the light pulses were detected by a piezoelectric transducer we designed ourselves, which consisted of a P(VdF/TrFE) film of 55 μm in thickness. Output signals of the transducer were amplified with a low-noise amplifier (bandwidth, 1 kHz–100 MHz; gain, 46 dB) and acquired with a multi-channel digital oscilloscope (bandwidth, 1 GHz). The relaxation time, calculated as the time for the thermoelastic wave amplitude to decrease by a factor of $1/e$, gave the relaxation parameters ($\eta/G$) of tissue, where $\eta$ is viscosity and $G$ is elasticity. Time-resolved fluorescent spectroscopy was obtained by a photonic multi-channel analyzer with intensified CCD. For time-resolved measurement, a trigger signal was controlled by a 4-channel digital signal generator. Fluorescent features of the developed measurement system are as follows: wavelength range, 200–860 nm; wavelength resolution $<3$ nm; exposure time, 19 milliseconds; gate time, 10 nanoseconds. The parameters of measured fluorescence, obtained using MatLab software, were peak wavelength at fluorescence maximum, fluorescent spectral bandwidth at half-maximal amplitude (FWHM), and integrated intensity of time-resolved spectrum. The figure was cited and modified from Bioengineering: Principles, Methodologies, and Applications ISBN: 978-1-60741-762-0 Authors: M. Sato et al., Editors: A. Garcia et al., pp. 179–190, 2010 Nova Science Publishers, Inc. Chapter 7.

## Safety Test

To assess the safety of the photoacoustic measurement method, we used a cell proliferative activity test in cultivated domestic rabbit chondrocytes and examined the effects on chondrocytes of laser beam irradiation to induce photoacoustic signals. As irradiation conditions of the laser were based on the third harmonic frequency of a Q switch Nd:YAG laser with a wavelength of 355 nm, the following five groups were established and examined: (1) a group treated under clinically used radiation conditions (100 μJ/mm$^2$, 30 shots, $n = 6$); (2) a group treated under conditions in which the pulse energy was 1.5-times greater than that used clinically (150 μJ/mm$^2$, 30 shots, $n = 6$); (3) a group treated under conditions in which the number of pulse shots was 50 times higher than the

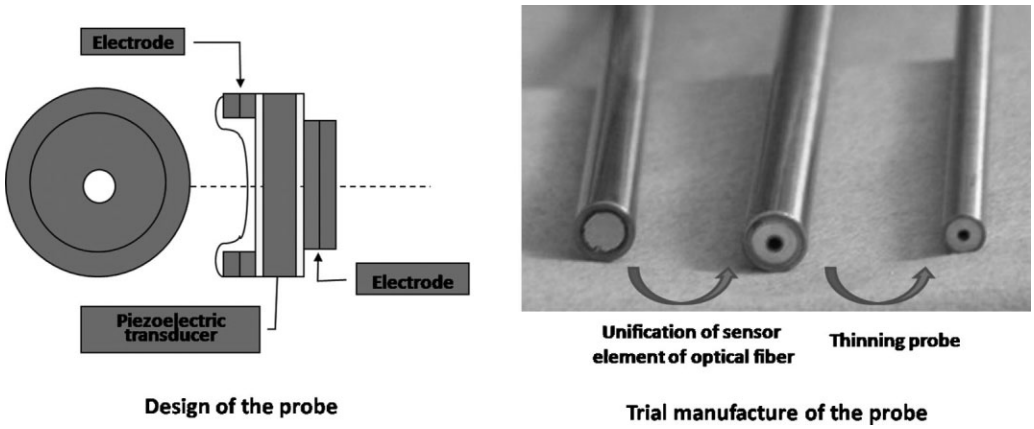



Fig. 3. Development of the probes. We developed a probe in which the optical output was introduced via a quartz glass optical fiber (core diameter 400 nm; Thorlabs Japan), and the P (VdF/TrFE) of a piezoelectric polymer film was used to detect the photoacoustic waves. In this polymer film, the laser irradiation side and the measuring side were originally opposite, so only transparent objects could be evaluated in vitro. However, with repeated trial and error, we developed an integrated optical fiber reflective probe that allowed measurements to be made in vivo, specifically during arthroscopy, by situating the probe at the center and placing the sensors peripherally around it in a circle. The figure was cited and modified from Bioengineering: Principles, Methodologies, and Applications ISBN: 978-1-60741-762-0 Authors: M. Sato et al., Editors: A. Garcia et al., pp. 179–190, 2010 Nova Science Publishers, Inc. Chapter 7.

number used clinically (150 $\mu$J/mm$^2$, 1,500 shots, $n = 6$); (4) a positive control group to which 70% ethanol was added to completely kill the cells ($n = 4$); and (5) a negative control with no laser irradiation ($n = 4$). Notably, the pulse energy used to treat group (2) represented the maximum output of this device. A WST-8 assay (Dojindo Laboratories, Kamimashiki, Kumamoto, Japan) was used for the cell proliferative activity test. We applied the abovementioned conditions to cultivated cells sown in a 96-well plate and cultured at 37°C under 5% $CO_2$, with all measurements made after 1 hour.

### Comparative Studies of Mechanical Properties for Tissue-Engineered Cartilage Measured by Photoacoustic Method and Intrinsic Viscoelastic Measurements

**Tissue-engineered cartilage made from chondrocytes cultured using scaffold.** Twelve knee joints were obtained from 4-week-old female Japanese White rabbits, each weighing about 1 kg. Articular cartilage was separated from the joint with a scalpel and digested for 4 hours in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 0.0125% (w/v) bacterial collagenase P (Roche, Mannheim, Germany) and 0.05% actinase E (Kaken Pharmaceutical, Tokyo, Japan). The digested tissue was passed through a cell strainer (BD Biosciences, Wobum, MA) with a pore size of 40 $\mu$m. The filtrate was centrifuged at 1,500 rpm for 10 minutes to separate the cells. Cells were then seeded at high density ($1 \times 10^6$ cells per scaffold) into an ACHMS scaffold (atelocollagen honeycomb with a membrane seal; diameter, 11 mm; thickness, 2 mm) [41,43,44], which we

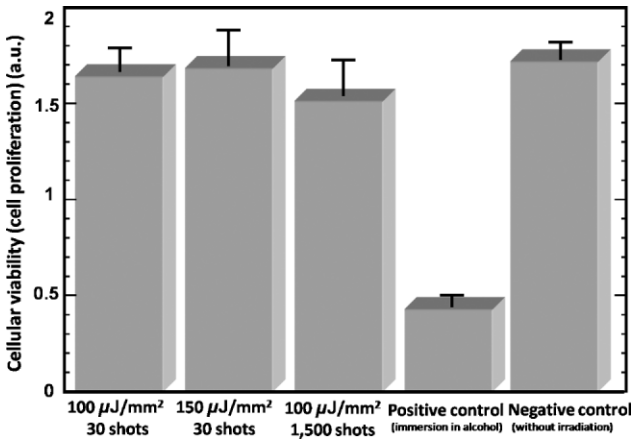



Fig. 4. Effect of laser irradiation (energy per mm$^2$) on cell viability (cell proliferation). Results of a cell proliferation assay using WST-8. Data were obtained 1 hour after incubation and inoculation. Group A: 100 mJ/mm$^2$, 30 shots (this condition is standard in our studies). Group B: 150 mJ/mm$^2$, 30 shots (this condition represents maximum fluence of this laser system). Group C: 100 mJ/mm$^2$, 1,500 shots. Group D: Positive control (immersion in alcohol). Group E: Negative control without irradiation. Error bars show standard deviation ($n = 4$–6). Results for Group E showed no significant differences from those for the other laser irradiation groups (Groups A–C). The figure was cited and modified from Lasers in Surgery and Medicine 38:249–255 (2006) Authors: M. Ishihara, M. Sato et al.

had developed for three-dimensional and high-density culture in 48-well plates (Sumitomo Bakelite, Tokyo, Japan) by centrifugation at 500 rpm for 5 minutes and then cultured in DMEM-F12 (Iwaki, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% $CO_2$ in air and 100% relative humidity. After the indicated periods of incubation, tissue-engineered cartilages using ACHMS scaffold (Fig. 5) were studied biomechanically using the photoacoustic method ($n = 6$) and intrinsic viscoelastic measurements ($n = 6$).

### Biomechanical Study

**Photoacoustic method.** The third harmonic frequency of a Q switch Nd:YAG laser (wavelength, 355 nm; pulse width, 5–6 nanoseconds; Excel Technology, Tokyo, Japan) was used at a constant repetition rate of 10 Hz. The beam was focused using a lens and then coupled to a silica fiber with a core diameter of 400 μm. Transmitted light energy was maintained at approximately 50 μJ/pulse. Stress waves induced by the light pulses were detected at the back surface of the sample by a piezoelectric transducer consisting of P(VdF/TrFE) film, 4 mm in diameter and 55 μm in thickness. Output signals of the photoacoustic transducer were amplified using a low-noise amplifier (bandwidth, 1 kHz–100 MHz; gain, 46 dB) and acquired with a multichannel digital oscilloscope (bandwidth, 1 GHz). Relaxation time $T$, which was calculated as the time required for stress wave amplitude to

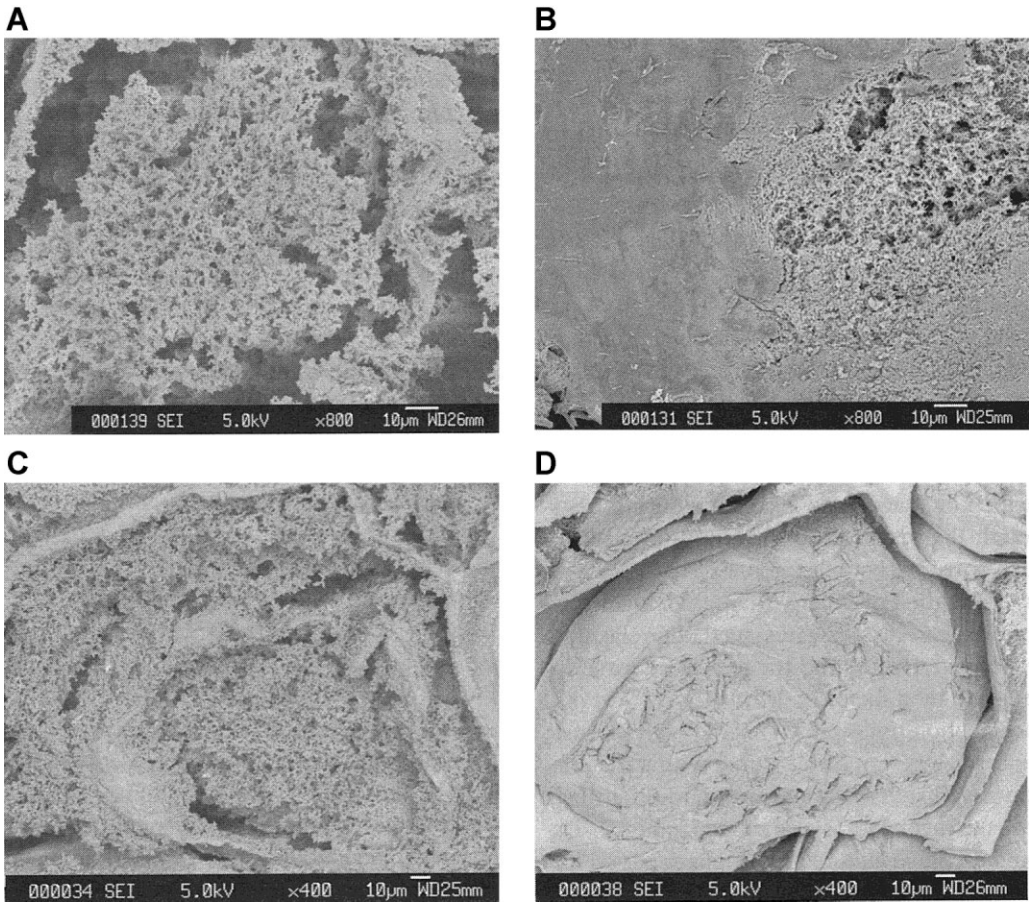



Fig. 5. Tissue-engineered cartilage using ACHMS scaffold. This figure shows a comparison of scanning electron microscopic (SEM) images of tissue-engineered cartilage cultured for 3 and 12 weeks. The lower magnification image of cartilage cultured for 3 weeks (**C**) shows a loose extracellular matrix in the honeycomb-shaped partition. In the higher-magnification image of cartilage cultured for 3 weeks (**A**), a network of collagen fibrils and interspersed proteoglycan is shown. In the images of cartilage cultured for 12 weeks (**B** and **D**), a tight extracellular matrix in the honeycomb partition is shown. Images differ considerably from those of cartilage cultured for 3 weeks, in which collagen and proteoglycan cannot be distinguished. Formation of an extracellular matrix is obvious. The figure was cited from Tissue Engineering Volume 11, Number 7/8, 2005, Mary Ann Liebert, Inc. Authors: M. Ishihara, M. Sato et al.

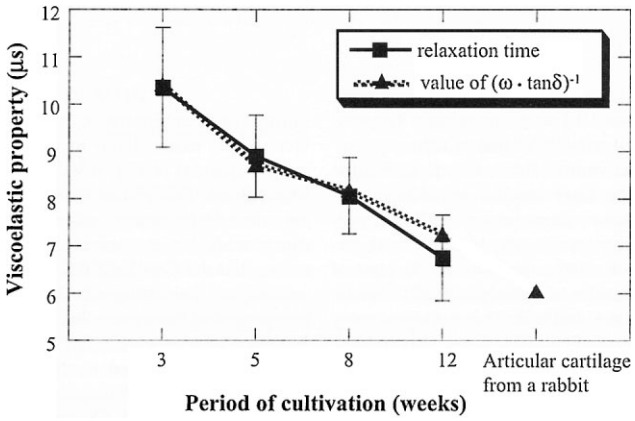



Fig. 6. Comparison of relaxation times measured by the photoacoustic method and values of $(\omega \cdot \tan \delta)^{-1}$ (intrinsic viscoelastic properties) measured with a rheometer as a function of culture time ($\omega = 0.75$ MHz). (■) Relaxation times; (▲) values of $(\omega \cdot \tan \delta)^{-1}$. The correlation coefficient is 0.98. Error bars indicate the standard deviation. To compare viscoelasticities of engineered tissue and native tissues, the value of $(\text{cotan } \delta)^{-1}$ for articular cartilage from a rabbit is plotted ($n = 6$). The figure was cited and modified from Tissue Engineering Volume 11, Number 7/8, 2005, Mary Ann Liebert, Inc. Authors: M. Ishihara, M. Sato et al.

decrease by a factor of $1/e$, was measured. Relaxation time shows a relationship with the viscous-to-elastic modulus rate ($\tan \delta$) when $\omega$ is defined as the frequency of a stress wave: $T = (\omega \cdot \tan \delta)^{-1}$ [31].

**Intrinsic viscoelastic measurements.** Intrinsic viscoelastic properties of the same samples as those used for the photoacoustic measurements were examined with a rheometer, a conventional viscoelastic analyzer, and the data were compared to those obtained using the photoacoustic method. Measurements using a rheometer were made at an environmental temperature of 20°C and an initial stress of 80 Pa [31].

The recorded waveform of the photoacoustic signal of the engineered cartilages cultured for 12 weeks is shown in the monitor of a digital oscilloscope in Figure 2. The signal shows a pulse sequence that is due to multiple acoustic reflections at the acoustic boundaries. When attenuation of the stress wave intensity is affected only by reflection at the boundaries and relaxation during transmission through the viscoelastic material, the peak intensity of each wave packet is expressed as an exponential function. Using the Levenberg–Marquardt algorithm, a nonlinear least-squares method, relaxation time can be derived as the time for the stress wave intensity to decrease by a factor of $1/e$.

**Evaluation of degenerated cartilage.** To produce experimentally degenerated cartilage, we created cartilage with different degrees of degradation by extracting 22 osteochondral plugs (diameter, 12 mm) from four swine patellar cartilage and processing them with trypsin (trypsin-1 × EDTA; Invitrogen, Carlsbad, CA) to cause an outflow of proteoglycan, reflecting changes in the mechanical characteristics of the tissue in vivo. Trypsin was applied for up to 24 hours at a concentration of 1 mg/ml. We assessed degenerated cartilage using the photoacoustic measurement method. After measurements had been made, samples were fixed in 10% formalin solution for histological study. Samples (0 hours: $n = 6$, 6 hours: $n = 6$, 12 hours: $n = 6$, 24 hours: $n = 4$) were sectioned to 4-μm thick slices for microscopic observation and stained with toluidine blue.

### Evaluation of Osteochondral Defects in Rabbit Articular Cartilage Using Photoacoustic Measurement Method

We demonstrated the capability of photoacoustic measurement for viscoelastic characterization. Since tissue viscoelasticity affects the propagation and attenuation of photoacoustic waves generated in the tissue, the relaxation times of the photoacoustic waves give the viscoelastic ratio of the tissue. The relaxation times of photoacoustic waves of articular cartilage tissues engineered under various culture conditions were closely correlated with intrinsic viscoelastic ratio measured by using a conventional viscoelastic analyzer ($R > 0.98$, Fig. 6). In order to apply the photoacoustic measurement method to evaluation of the regeneration of articular cartilage as a method to validate the surgery, the method should enable not only evaluation of engineered tissue during cultivation in vitro but also evaluation after transplantation of engineered tissue in vivo. We performed regenerative medicine using the rabbit osteochondral defect model and tissue engineered cartilage using ACHMS scaffold ($n = 8$, Fig. 5).

### Evaluation of Cartilage Using Time-Resolved Autofluorescence Spectroscopy

For time-resolved autofluorescence spectroscopy, we used the third harmonic frequency of the Q switch Nd:YAG laser for the excitation light introduced via an optic fiber, in a manner similar to that used in the photoacoustic measurement method. We used a charge-coupled device (CCD) sensor with an image intensifier as the photodetector, while controlling the spectroscopic system that could be measured by a nanosecond order with a 4-channel digital pulse generator. Fluorescence peak intensity, half bandwidth, peak wavelength, fluorescence volume, and fluorescence life were calculated as the measurement parameters. The articular cartilage of Japanese white domestic rabbits ($n = 4$), the outer layer of the annulus fibrosus ($n = 4$), and commercially available type I and type II collagen (powder; Ieda Chemical, Tokyo, Japan, $n = 4$, respectively) were used as the target samples.

## RESULTS

### Determination of Optimal Wavelength

Photoacoustic signals could thus be measured at any wavelength within this range [29,31]. The shorter wavelengths within this range can magnify absorption by

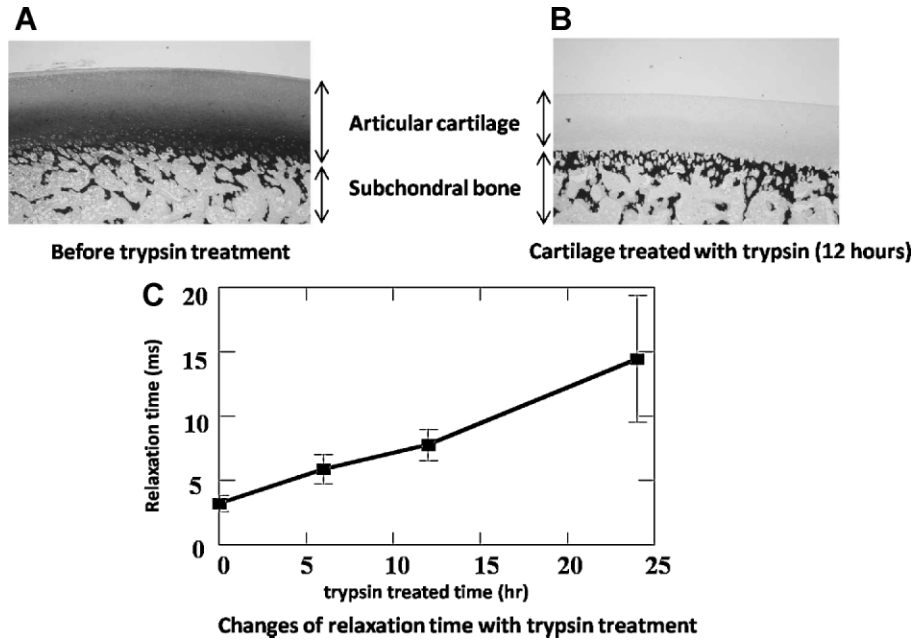



Fig. 7. Photoacoustic evaluation of characteristic viscoelastic changes with cartilage degeneration. Trypsin treatment of the tissue caused marked loss of proteoglycans in the cartilage, as shown in **A** and **B**. (a) Toluidine blue staining of a normal porcine cartilage specimen. (b) Toluidine blue staining of a cartilage specimen treated with trypsin for 12 hours. The specimen shows an extensive loss of proteoglycans in the tissue. Changes in the extracellular matrix simultaneously caused change in viscoelasticity of the cartilaginous tissue. The degree of change in viscoelasticity corresponded to the degree of change in the extracellular matrix. **C**: Relaxation times measured by the photoacoustic measurement method as a function of trypsin treatment time (hours). Error bars show standard deviation (0 hours: $n = 6$, 6 hours: $n = 6$, 12 hours: $n = 6$, 24 hours: $n = 4$). The figure was cited and modified from Lasers in Surgery and Medicine 38:249–255 (2006) Authors: M. Ishihara, M. Sato et al.

living organs, so peak values of the initiated photoacoustic waves can be increased and the initiation depth of the photoacoustic wave set at a shallower level. However, in practical terms, a small, portable, and inexpensive excitation light source is desirable, so we devised a system in which the third harmonic frequency of a Q switch Nd:YAG laser (wavelength, 355 nm; pulse width, 5–6 nanoseconds; Excel Technology) was used [39,40]. We developed a probe in which the optical output was introduced via a quartz glass optical fiber (core diameter, 400 nm; Thorlabs Japan, Tokyo, Japan), and the poly(polyvinylidene fluoride) copolymer (P(VdF/TrFE)) of a piezoelectric polymer film (Nishiki Trading Company, Tokyo, Japan) was used to detect the photoacoustic waves [40]. In this polymer film, the laser irradiation side and measuring side were originally opposite, so only transparent objects were able to be evaluated in vitro. However, with repeated trial and error, we developed an integrated optical fiber reflective probe that allowed measurements to be taken in vivo, specifically during arthroscopy, by situating the probe at the center and placing the sensors peripherally around the probe in a circle (Fig. 3).

### Effect of Laser Irradiations

We confirmed a lack of significant differences between any laser-irradiated group and the non-irradiated group, and found that laser irradiation in this study had no effect on cell proliferative activity (Fig. 4) [40].

### Mechanical Properties of Tissue-Engineered Cartilage Measured by Photoacoustic Method and Intrinsic Viscoelastic Measurements

In Figure 6, relaxation times are compared to intrinsic viscoelastic parameters ($\tan \delta$) measured with a rheometer. The intrinsic relaxation parameter of native cartilage measured with the rheometer is also plotted in Figure 6. Tissue-engineered cartilage cultured for a longer period showed smaller relaxation times. The relaxation times obtained by photoacoustic measurement agreed well with the measured intrinsic relaxation parameters, with a correlation coefficient of 0.98. Compared to native cartilage,

cartilage cultured for the longest period (12 weeks) showed an 85% smaller viscoelastic parameter [31].

### Photoacoustic Evaluation of Characteristics of Degenerated Cartilage

Figure 7 shows the positive correlation between damping time and trypsinization time [40]. Specifically, damping time increased with increasing trypsinization time. In other words, viscosity increased and elasticity decreased. Histologically, the stainability of tissue with toluidine blue also decreased with trypsinization and the loss of proteoglycans, suggesting that the course of tissue changes involved in cartilage degeneration can be monitored using the photoacoustic measurement method.

### Monitoring the Post-Operative Regenerative Process of Articular Cartilage Using Photoacoustic Measurement Method

We confirmed that the usefulness of the photoacoustic method for repeated measurement of viscoelastic properties of regenerative articular cartilage after allografted tissue-engineered cartilage. The photoacoustic measurements enabled the determinations of viscoelasticities of regenerative cartilage during the total time course after surgery (Fig. 8).

### Compositional Information of Cartilage Using Time-Resolved Autofluorescence Spectroscopy

The articular cartilage exhibited a spectrum close to that of type II collagen, and peak wavelengths and half bandwidths were also similar [33,38]. Conversely, the outer layer of the annulus fibrosus exhibited a spectrum close to that of type I collagen, and peak wavelengths and half bandwidths were also similar (Fig. 10) [42,43]. This indicates that the collagen composition of tissue can be measured, as collagen is an autofluorescent substance used in vivo in a non-contact manner. This is significant, as the content ratio of type I to type II collagen is particularly important in diagnosing the degree of cartilage degradation.

## DISCUSSION

Based on the above results, we applied the photoacoustic measurement method to evaluate the articular cartilage under the arthroscopy. We received the approval of the Institutional Review Board of Tokai University Hospital concerning the photoacoustic measurement method, and applied the method to some kinds of arthroscopic surgeries. The surgeon can easily have a true figure of photoacoustic waveform using the real-time monitoring. The measuring photoacoustic waveform in the monitor can be changed to make larger or smaller by switching (Fig. 9). The measurable thickness was limited from approximately 1.5 to 6 mm in the present experimental condition. In this range of the cartilage thickness, the effect of the thickness on the accuracy of the measurement was ignorable.

Many elderly people who suffer from lifestyle-related diseases are also affected by osteoarthritis and are often unable to perform exercises that would normally be within their physical capacity, due to joint pain and limited range of motion. This is particularly serious in patients with diabetes, hyperlipidemia, or obesity, and the disease may be exacerbated because osteoarthritis reduces the ability to exercise, even when exercise therapy is available. In osteoarthritis, evaluating the prognosis of conservative therapy or the treatment effects after surgery often depend on the symptoms of the

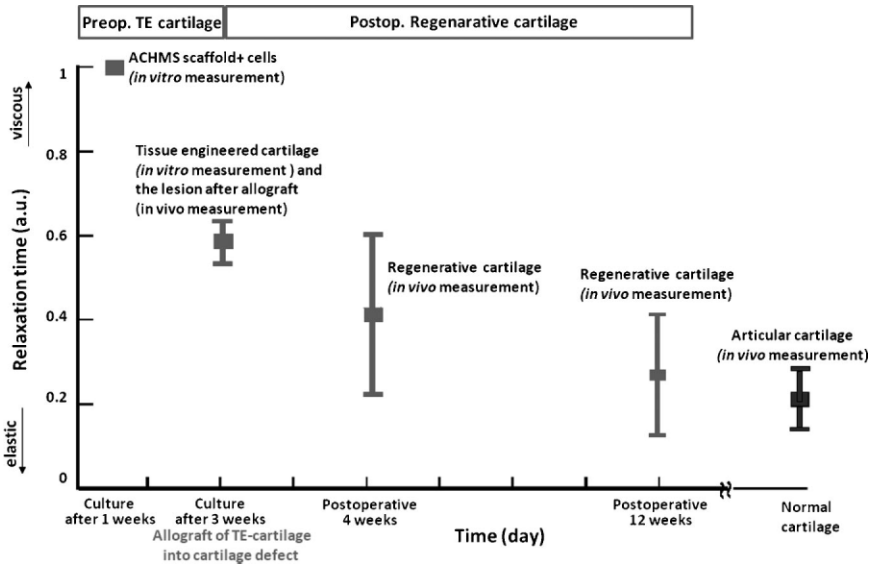



Fig. 8. The results of photoacoustic measurement of pre- and post-operative cartilage. A total time course of pseudo-regenerative medicine using rabbit model was able to be monitored by photoacoustic method.

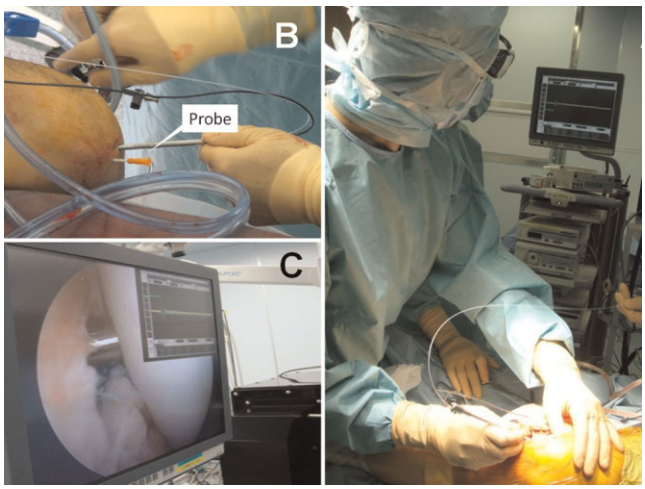
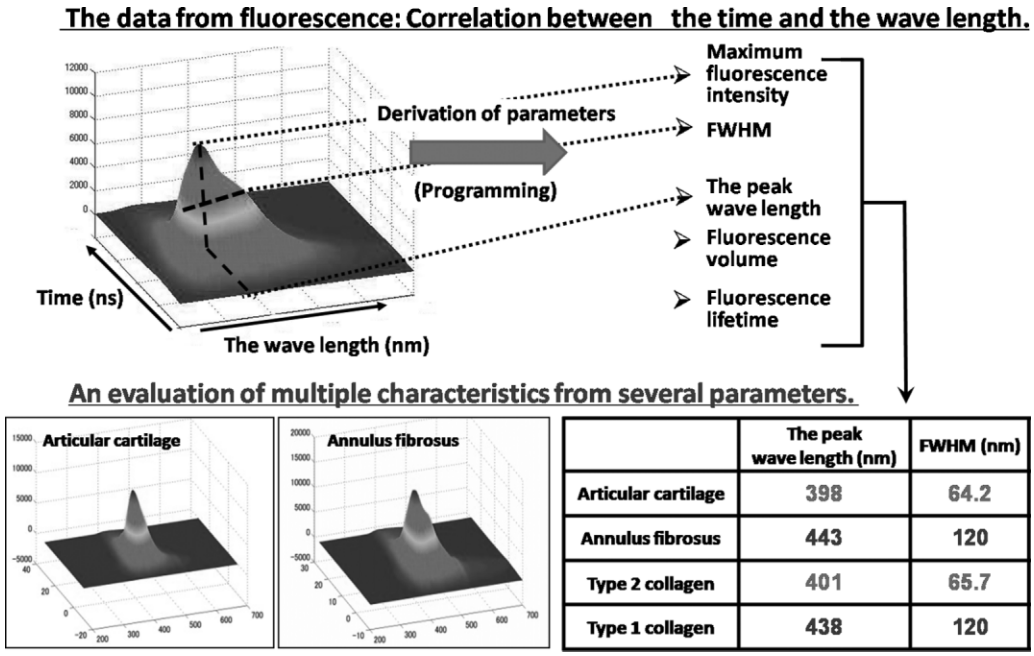



Fig. 9. Clinical application of photoacoustic measurement. **A**: The measuring photoacoustic waveform can be checked by the monitor of arthroscopy. **B**: The integrated optical fiber of reflective probe was 4mm in diameter (Fig. 3). **C**: The measuring photoacoustic waveform in the monitor can be changed to make larger or smaller by switching.

patient, so the pathological condition is not accurately understood. Surgical treatments such as artificial joint replacement are currently performed on patients in the terminal phase, whereas patients in the initial to middle phases are treated conservatively, often without any clear aims.

The present studies have demonstrated that the mechanical characteristics and properties of articular cartilage can be evaluated simultaneously during arthroscopy using a non-invasive intense pulsed laser (Table 1). We are now developing a device for this application by trial and error. If such a device is developed, accurate measurement of the mechanical characteristics involved with the original function of the articular cartilage and the associated tissue properties will be possible during arthroscopy, and anyone could perform such quantitative functional evaluations. This will enable an accurate understanding of the pathological features of osteoarthritis and careful planning and implementation of treatments. This technology could also allow quantitative measurement and evaluation of mechanical characteristics and tissue properties simultaneously, to assess treatment effects such as those of a variety of drugs, in




| | The peak wave length (nm) | FWHM (nm) |
|---|---|---|
| Articular cartilage | 398 | 64.2 |
| Annulus fibrosus | 443 | 120 |
| Type 2 collagen | 401 | 65.7 |
| Type 1 collagen | 438 | 120 |

Fig. 10. Analyses with time-resolved autofluorescence spectroscopy. Fluorescence peak intensity, full width at half maximum (FWHM), peak wavelength, fluorescence volume, and fluorescence life were calculated as the measurement parameters. The articular cartilage of Japanese white domestic rabbits, the outer layer of the annulus fibrosus, and commercially available type I and type II collagen were used as target samples. Articular cartilage exhibited a spectrum close to that of type II collagen, and peak wavelengths and half bandwidths were also similar. Conversely, the outer layer of the annulus fibrosus exhibited a spectrum close to that of type I collagen, and peak wavelengths and half bandwidths were also similar. This indicates that the collagen composition of tissue can be measured, as collagen is an autofluorescent substance used in vivo in a non-contact manner. The figure was cited and modified from Bioengineering: Principles, Methodologies, and Applications ISBN: 978-1-60741-762-0 Authors: M. Sato et al., Editors: A. Garcia et al., pp. 179–190, 2010 Nova Science Publishers, Inc. Chapter 7.

addition to the conventional evaluation of clinical symptoms such as pain or inflammation around the joints. We believe that this methodology will be useful in the objective evaluation of articular cartilage in investigations such as clinical trials of new drugs. This diagnostic system is a methodology used during arthroscopy, and so cannot be a completely non-invasive evaluation [41]. However, if quantitative data are collected during arthroscopy treatments, the effects of a variety of conservative therapies will be able to be predicted, based on the severity of cartilage degeneration. Planning and performance of treatments on an individual basis will thus be possible. Accordingly, we are certain that the development of this technology and practical diagnostic devices will improve activities of daily living and quality of life for patients, and thus contribute to a healthy life expectancy.

## CONCLUSION

1. A photoacoustic measurement method using a non-invasive nanosecond-pulsed laser allows evaluation of the mechanical characteristics of cartilage, and time-resolved autofluorescence spectroscopy allows the evaluation of tissue properties for analysis.
2. This measurement system, based on interactions between optics and living organs, is an evaluation methodology suitable for making diagnoses during arthroscopy.

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