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 (η/G) of the tissue, where η 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

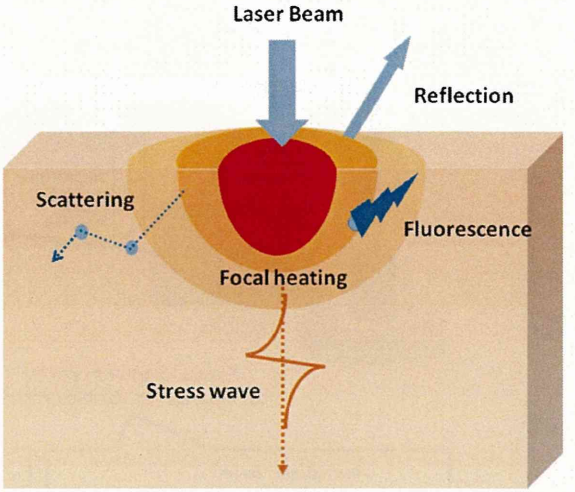


Fig. 1. Mutual interaction between light and a living body. The scattering, reflection, and increase in temperature attributable to absorption and the production of fluorescence and acoustic waves are regarded as the main effects when light or laser beams irradiate living organs to be measured. The figure 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 (τ) 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_δ is the time after laser irradiation, and τ 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.

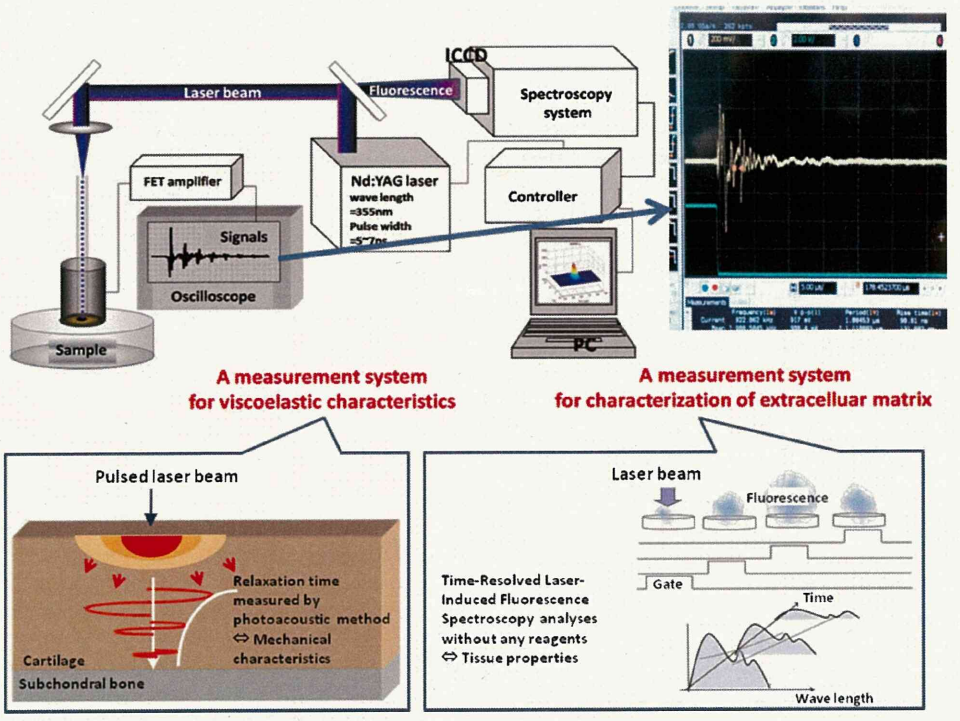


Fig. 2. Simultaneous measurement system: photoacoustic measurement of viscoelastic characteristics and fluorescent measurement with time-resolved autofluorescence spectroscopy. From a practical perspective, a commercially available 3rd (355 nm) harmonic Q-switched Nd:YAG laser (pulse width, 5–6 nanoseconds) 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 (η/G) of tissue, where η 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

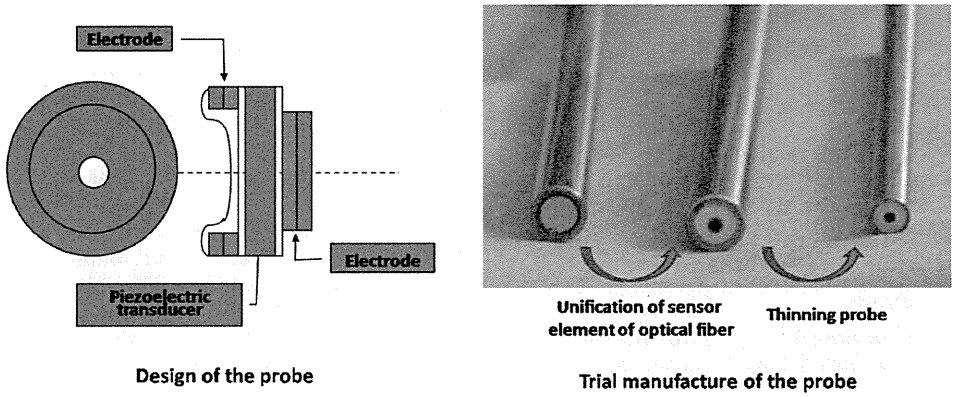


Fig. 3. Development of the probes. We developed a probe in which the optical output was introduced via a quartz glass optical fiber (core diameter 400 nm; Thorlabs Japan), and the P (VdF/TrFE) of a piezoelectric polymer film was used to detect the photoacoustic waves. In this polymer film, the laser 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 μJ/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 μm. The filtrate was centrifuged at 1,500 rpm for 10 minutes to separate the cells. Cells were then seeded at high density (1×10^6 cells per scaffold) into an ACHMS scaffold (atelocollagen honeycomb with a membrane seal; diameter, 11 mm; thickness, 2 mm) [41,43,44], which we

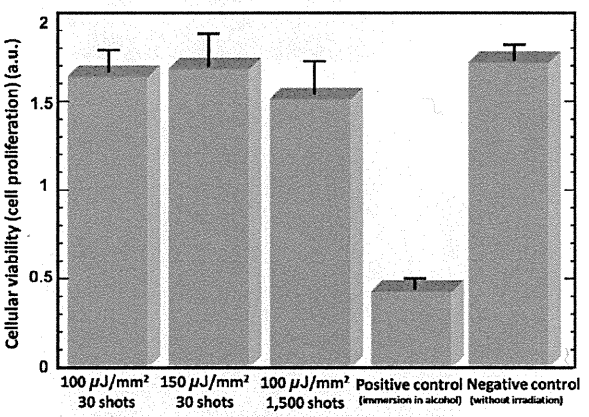


Fig. 4. Effect of laser irradiation (energy per mm^2) on cell viability (cell proliferation). Results of a cell proliferation assay using WST-8. Data were obtained 1 hour after incubation and inoculation. Group A: 100 mJ/mm^2, 30 shots (this condition is standard in our studies). Group B: 150 mJ/mm^2, 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

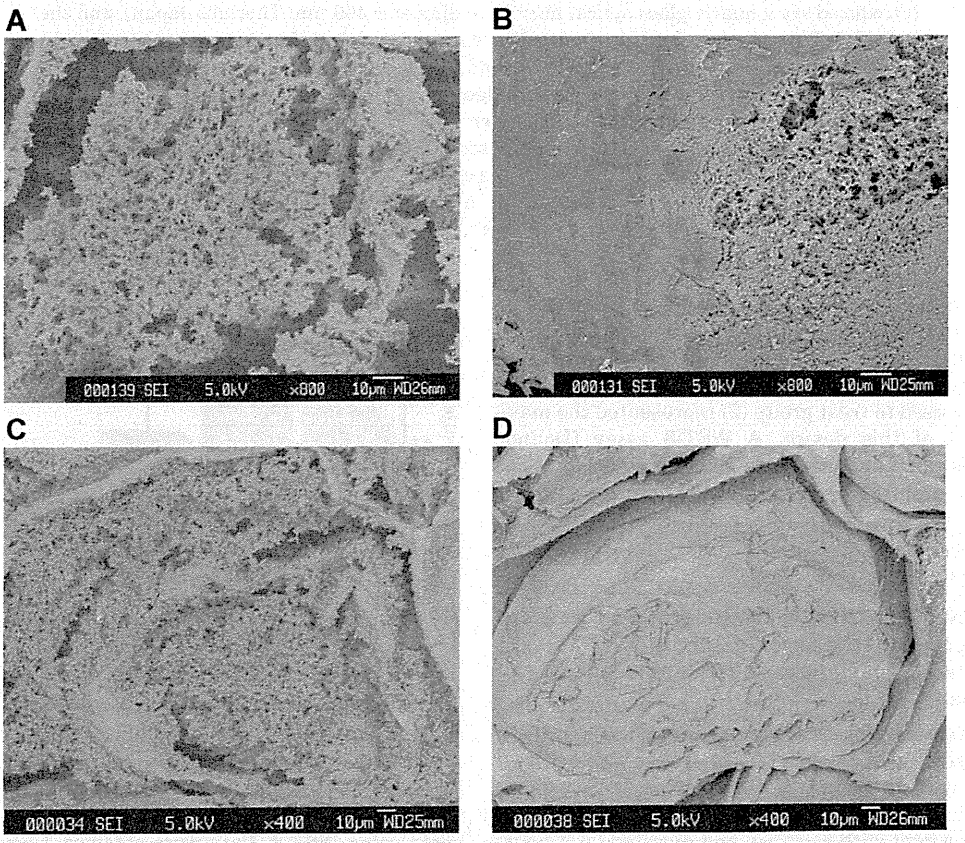


Fig. 5. Tissue-engineered cartilage using ACHMS scaffold. This figure shows a comparison of scanning electron microscopic (SEM) images of tissue-engineered cartilage cultured for 3 and 12 weeks. The lower magnification image of cartilage cultured for 3 weeks (**C**) shows a loose extracellular matrix 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.

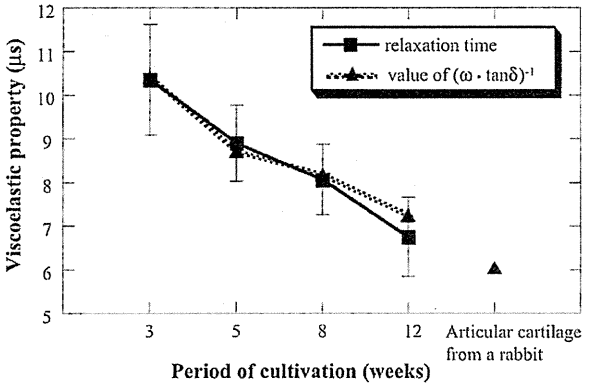


Fig. 6. Comparison of relaxation times measured by the photoacoustic method and values of $(\omega \cdot \tan \delta)^{-1}$ (intrinsic viscoelastic properties) measured with a rheometer as a function of culture time ($\omega = 0.75$ MHz). (■) Relaxation times; (▲) values of $(\omega \cdot \tan \delta)^{-1}$. 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 δ) when ω 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

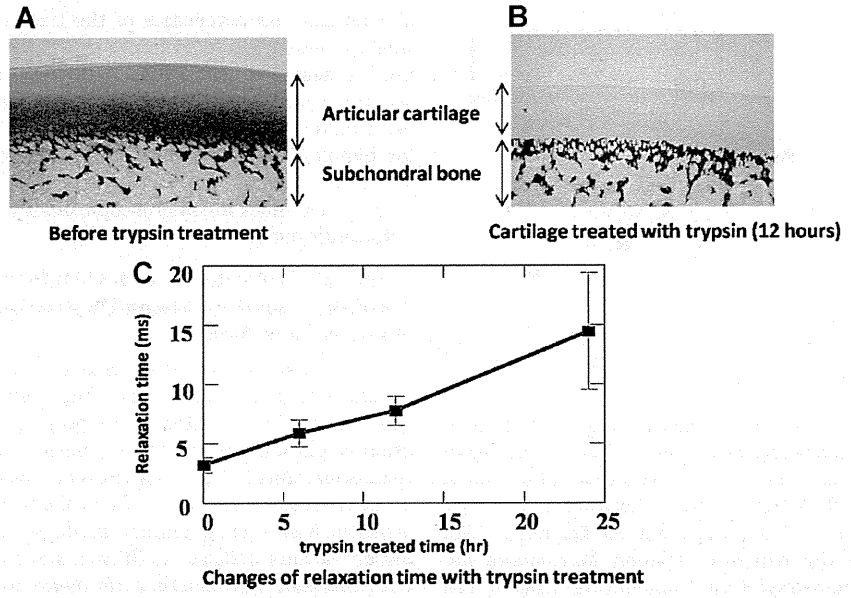


Fig. 7. Photoacoustic evaluation of characteristic viscoelastic changes with cartilage degeneration. Trypsin treatment of the tissue caused marked loss of proteoglycans in the cartilage, as shown in **A** and **B**. (a) Toluidine blue staining of a normal porcine cartilage specimen. (b) Toluidine blue 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 δ) 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

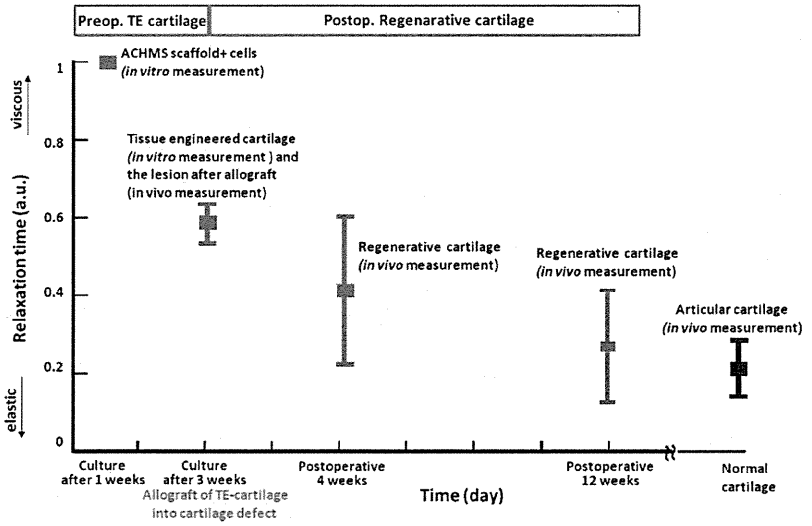


Fig. 8. The results of photoacoustic measurement of pre- and post-operative cartilage. A total time course of pseudo-regenerative medicine using rabbit model was able to be monitored by photoacoustic method.

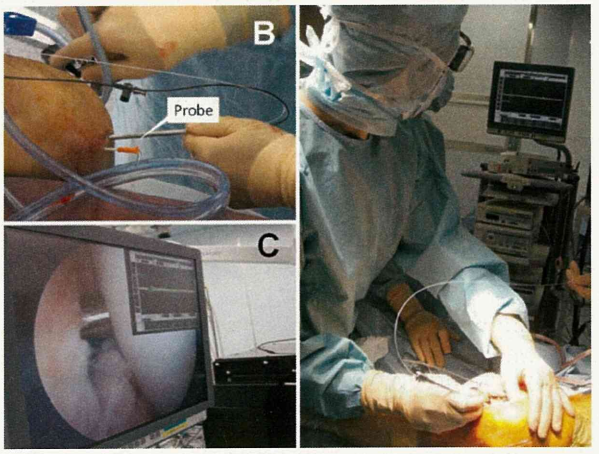
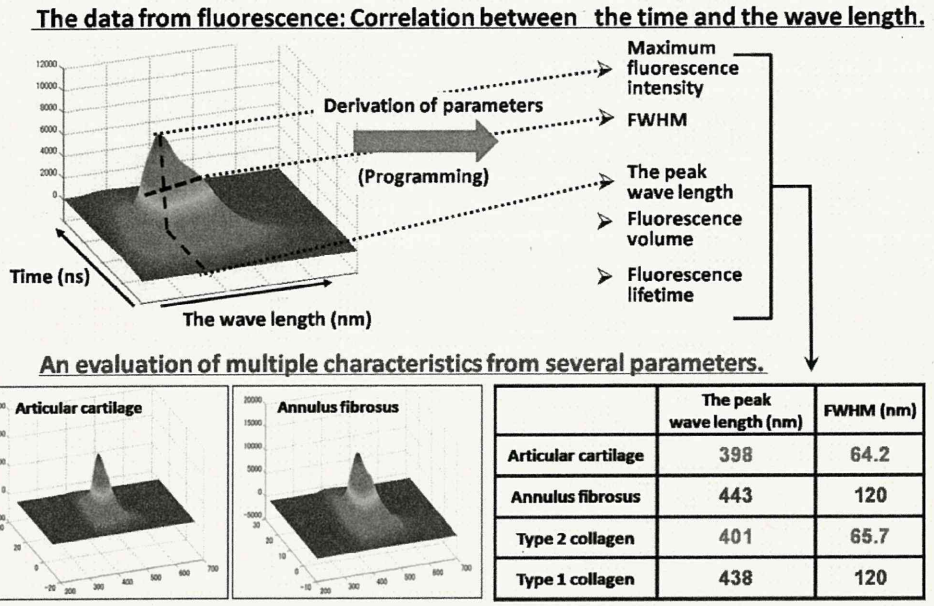


Fig. 9. Clinical application of photoacoustic measurement. **A**: The measuring photoacoustic waveform can be checked by the monitor of arthroscopy. **B**: The integrated optical fiber of reflective probe was 4mm in diameter (Fig. 3). **C**: The measuring photoacoustic waveform in the monitor can be 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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プログラム・抄録集

Tokyo, April 29-May 1, 2011

4月30日(土)　午後の部

特別講演

特別講演2　　30日(土)　12:50－13:35　A会場　P.196

座長：梶谷 文彦（川崎医療福祉大学）

Ecosystems for Personal Health Systems and Personal Health Record
Niilo Saranummi（VTT Technical Research Centre of Finland）

特別講演3　　30日(土)　13:35－14:20　A会場　P.196

座長：林 紘三郎（岡山理科大学）

Regenerative Medicine: The Past, the Present, and Unlocking the Future
Robert M. Nerem（Institute Professor and Director, Georgia Tech/Emory Center for Regenerative Medicine, Atlanta, Georgia, U.S.A.
Distinguished Visiting Professor, Chunbuk National University, Jeonju, Korea）

特別シンポジウム

特別シンポジウム　　30日(土)　14:30－16:00　A会場　P.197

「大震災対応特別シンポジウム」
"Special Symposium on BME in Japan Huge Disasters"

シンポジウム

シンポジウム2-1　　30日(土)　14:30－16:00　F会場　P.197

S2-1　「再生組織の非侵襲計測を目指して」
"Towards non-invasive measurements of regenerated tissues"
オーガナイザー・座長：牛田 多加志（東京大学）

S2-1-1　光音響原理と分光特性を利用した関節軟骨再生の評価
石原 美弥（防衛医科大学校 医用工学講座）
Photoacoustic measurement technology in regenerative medicine of articular cartilage
Miya Ishihara（Dept. of Medical Engineering, National Defense Medical College, Saitama, Japan）

S2-1-2　テラヘルツ分光法による再生軟骨のマトリックス・水分子相互作用の非侵襲計測の試み
古川 克子（東京大学大学院工学系研究科 バイオエンジニアリング専攻・機械工学専攻）
Non-invasive measurement of ECM-water interactions of regenerative cartilage tissue by terahertz technology
Katsuko Furukawa（Department of Bioengineering, and Mechanical Engineering, School of Engineering, University of Tokyo, Japan）

O3-7　「光学計測Ⅱ」
"Optical Measurement II"

座長：石原 美弥（防衛医科大学校）
大川 晋平（電気通信大学）

O3-7-1　**血管透視像の分光解析による動静脈判別の試み**
松田 康志（北海道大学大学院 情報科学研究科）
Attempt for arteriovenous discrimination by spectroscopic analysis of transillumination images
Yasushi Matsuda（Graduate School of Information Science and Technology, Hokkaido University, Sapporo, Japan）

O3-7-2　**多点励起による蛍光体深さ推定の試み**
夏　棘驁（北海道大学大学院 情報科学研究科）
Attempt for depth estimation of fluorescent object using multiple excitation sources
Jiao Xia（Graduate School of Information Science and Technology, Hokkaido University, Sapporo, Japan）

O3-7-3　**多波長光源を用いた静脈透視画像改善**
西田 浩平（北海道大学大学院 情報科学研究科）
Improvement of transillumination image of blood vessels using multiple wavelength of light
Kohei Nishida（Graduate School of Information Science and Technology, Hokkaido University, Sapporo, Japan）

O3-7-4　**マウスを用いた蛍光トモグラフィーのin vivo測定**
三井 陽平（電気通信大学大学院情報理工学研究科 知能機械工学専攻）
In vivo experiments of fluorescence diffuse optical tomography using mice
Yohei Mitsui（The University of Electro-Communications, Tokyo, Japan）

O3-7-5　**深部組織の高分解能画像化に向けた光音響画像診断法の開発と評価**
平沢　壮（防衛医科大学校 医用工学講座）
Development and evaluation of photo-acoustic imaging method for high resolution deep tissue imaging
Takeshi Hirasawa（Dept. of Medical Engineering, National Defense Medical College）

O3-7-6　**体外補助循環路を流れる血液の光吸収・散乱モデルの開発及びヘマトクリット・ヘモグロビンの非侵襲連続診断**
迫田 大輔（東京医科歯科大学 生体材料工学研究所）
Photon-cell interactive Monte Carlo (pciMC) simulation for non-invasive quantification of hematocrit and hemoglobin during extracorporeal circulation
Daisuke Sakota（Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan）

特別シンポジウム　14:30－16:00　A会場

大震災対応特別シンポジウム
Special Symposium on BME in Japan Huge Disasters

シンポジウム2「再生組織の非侵襲計測を目指して」　14:30－16:00　F会場

S2-1-1　光音響原理と分光特性を利用した関節軟骨再生の評価

○石原 美弥[1]、佐藤 正人[2]、沓名 寿治[2]、持田 譲治[2]、菊地　眞[1]
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Photoacoustic measurement technology in regenerative medicine of articular cartilage

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There is a demand in the field of regenerative medicine of articular cartilage for noninvasive measurement technology that enables determination of functions and components of engineered tissue. As the major function of articular cartilage is viscoelastic property, the photoacoustic measurement method may be a strong candidate for the required technology. Extracellular matrix of articular cartilage is responsible for the major functional properties of cartilage. Consequently, characterization of extracellular matrix should be performed. To meet this demand, we developed a measurement method of viscoelastic property using photo-acoustic temporal waveform and extracellular matrix characterization using time-resolved auto-fluorescence spectroscopy.The viscoelastic properties enabled to be determined and the status of extracellular matrix formation enabled to be provided when engineered articular cartilage cultured for various periods were used as samples. We demonstrated possibility of monitoring of the repair process after transplantation and diagnosis of a disease before regenerative medicine.

O3-7-5 深部組織の高分解能画像化に向けた光音響画像診断法の開発と評価

○平沢 壮[1]、石原 美弥[1]、辻田 和宏[2]、入澤 覚[2]、北垣 学[1]、藤田 真敬[3]、菊地 眞[1]
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Development and evaluation of photo-acoustic imaging method for high resolution deep tissue imaging

○Takeshi Hirasawa[1], Miya Ishihara[1], Kazuhiro Tsujita[2], Kaku Irisawa[2], Manabu Kitagaki[1], Masanori Fujita[3], Makoto Kikuchi[1]
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Photo-acoustic imaging (PAI) is an emerging functional imaging method, in which, optical absorbers in biological tissue such as hemoglobin are illuminated by nanosecond pulse laser to produce broad band ultrasound then, the ultrasound is detected by piezoelectric sensors to form a tomography. Although PAI can provide high resolution image at depth exceeding the limitation of conventional optical technique, the penetration depth is restricted by both optical scattering and acoustic attenuation, and the resolution is restricted by specification of acoustic sensor such as directionality, frequency band width, and aperture. Therefore, in this research, penetration depth and resolution of PAI were analyzed. Furthermore, PAI of arteriolar phantoms placed in highly optical scattering ambient medium was demonstrated to show the validity of this method. As a result, arteriolar phantoms placed few centimeters away from detector surface were imaged in sub-millimeter resolution. Clinical application for superficial organs and vascular could be considered.

O3-7-6 体外補助循環路を流れる血液の光吸収・散乱モデルの開発及びヘマトクリット・ヘモグロビンの非侵襲連続診断

○迫田 大輔、高谷 節雄
東京医科歯科大学 生体材料工学研究所

Photon-cell interactive Monte Carlo (pciMC) simulation for non-invasive quantification of hematocrit and hemoglobin during extracorporeal circulation

○Daisuke Sakota, Setsuo Takatani
Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan

We have sought for the non-invasive diagnosis of blood during the extracorporeal circulation support. To achieve the goal, we have developed a newly photon-cell interactive Monte Carlo "pciMC" model for optical propagation through blood. pciMC actually describes 3-dimentional biconcave RBCs. The scattering is described by micro-scopical RBC boundary condition based on geometric optics. By using pciMC, we modeled the RBCs inside the extracorporeal circuit will line-up in the direction of flow. The RBCs' orientation was defined as their long axis being parallel with flow direction. Simultaneously the RBCs were allowed to randomly rotate about flow direction. As a result, as flow rate increased, orientation rate increased and converged to approximately 20% at 1 L/min flow rate and over. And finally, by using this model, pciMC non-invasively and absolutely predicted hematocrit and hemoglobin at the accuracies of 0.97 [Hct%] and 0.32 [g/dL] respectively against measurements by a blood gas analyzer.

Photoacoustic Measurement Technology in Regenerative Medicine of Articular Cartilage

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Abstract: We developed a measurement method of viscoelastic property using photo-acoustic temporal waveform and extracellular matrix characterization using time-resolved auto-fluorescence spectroscopy. The viscoelastic properties enabled to be determined and the status of extracellular matrix formation enabled to be provided when engineered articular cartilage cultured for various periods were used as samples. We demonstrated possibility of monitoring of the repair process after transplantation and diagnosis of a disease before regenerative medicine.

Keywords: articular cartilage, viscoelastic property, extracellular matirix

1. Introduction

There is a demand in the field of regenerative medicine of articular cartilage for noninvasive measurement technology that enables determination of functions and components of engineered tissue.

As the major function of articular cartilage is viscoelastic property, the photoacoustic measurement method may be a strong candidate for the required technology.

Extracellular matrix of articular cartilage is responsible for the major functional properties of cartilage. Consequently, characterization of extracellular matrix should be performed.

To meet this demand, we developed a measurement method of viscoelastic property using photo-acoustic temporal waveform and extracellular matrix characterization using time-resolved auto-fluorescence spectroscopy.

2. Photoacoustic measurement and autofluorescence measurement

We discovered that stress wave propagation and attenuation by pulsed laser irradiation influenced tissue viscoelasticity, and based on this principle, proposed the use of photoacoustic measurement for viscoelastic characterization of biological tissue [1-2]. As the major function of articular cartilage is viscoelastic property, the photoacoustic measurement method may be a strong candidate for the required technology [3-5].

Extracellular matrix of articular cartilage is responsible for the major functional properties of cartilage. Consequently, characterization of extracellular matrix should be performed for evaluation of regenerative medicine for articular cartilage. Articular cartilage is composed of scattered chondrocytes embedded in an abundant extracellular matrix. The matrix is mainly composed of proteoglycans and type II collagen, which consists of endogenous biomolecules. Fluorescence measurement can provide information about endogenous fluorescent biomolecules.

3. Results and discussion

The viscoelastic properties enabled to be determined by the photoacoustic method when engineered articular cartilage cultured for various periods (up to 12 weeks) were used as samples. The photoacoustic measurement was also revealed to provide information about the status of extracellular matrix formation during cultivation. In vivo photoacoustic measurements were demonstrated to verify the usefulness for repeated measurement of viscoelastic properties in order to evaluate the process of regeneration after transplantaion tissue-engineered cartilage. About a 40% difference between the viscoelasticity of allografted engineered cartilage that of surrounding native tissue was shown just after surgery. The difference was significantly reduced at 4 and 12 postoperative weeks. Therefore, since the photoacoustic measurement method enables assessment of the progress of restoration of the viscoelasticity of articular cartilage, its main function, this method would be useful as an evaluation method in regener.

There were significant differences in the measured fluorescent parameters among the culture conditions of cartilage because chondrocytes produce a specific extracellular matrix depending on its culture condition. The constituents of the extracellular matrix of hyaline cartilage are different from those of the extracellular matrix of fibrous cartilage. Discrimination of hyaline cartilage and fibrous cartilage enables characterization of the regenerated articular cartilage because one of the serious problems in regenerative medicine of articular cartilage is that repaired osteochondral defect using tissue-engineering technology should be regenerated as hyaline cartilage, however it has been often regenerated as fibrous cartilage.

4. Conclusion

We developed the measurement method for the evaluation of the viscoelastic properties by the photoacoustic method and characterization of the extracellular matrix of tissue engineered cartilage by the time-resolved auto-fluorescence spectroscopy. Both of them are expected to become a useful evaluation method in regenerative medicine of articular cartilage.

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深部組織の高分解能画像化に向けた光音響画像診断法の開発と評価

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Development and evaluation of photo-acoustic imaging method for high resolution deep tissue imaging

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Abstract: Photo-acoustic imaging (PAI) is an emerging functional imaging method, in which, optical absorbers in biological tissue such as hemoglobin are illuminated by nanosecond pulse laser to produce ultrasounds then, ultrasounds are detected by piezoelectric sensors to form a tomography. Although PAI can provide high resolution image at depth exceeding the limitation of conventional optical techniques, the penetration depth is restricted by both optical scattering and acoustic attenuation, and the resolution is restricted by specifications of acoustic sensor such as directionality, frequency band width, and aperture. In this research, penetration depth and resolution of PAI were experimentally analyzed. Furthermore, PAI of arteriolar phantoms placed in highly optical scattering ambient medium was demonstrated to show the validity of this method. As a result, arteriolar phantoms placed few centimeters away from detector surface were imaged in sub-millimeter resolution. Clinical application for superficial organs and vascular could be considered.
Keywords: Photoacoustic, Optoacoustic, Thermoacoustic, Medical Imaging, Ultrasound, P(VDF-TrFE),

1. Introduction

Photo-acoustic imaging (PAI) is a relatively new diagnostic imaging technique using photo-acoustic (PA) effect. In PA effect, optical absorbers in biological tissue illuminated by pulsed laser generate ultrasounds due to thermo-elastic expansion [1]. Ultrasounds produced by optical absorbers propagate through biological tissue and then, detected by acoustic sensor consists of piezoelectric elements. The distance between an optical absorber and an acoustic sensor can be calculated from arrival time of signal. Furthermore, PA image which reflects optical properties of tissue can be obtained from PA signals measured at multiple positions. By scanning single sensor or using array sensor with multiple elements, those signals can be obtained.

PAI can provide high resolution image at depth exceeding the limitation of conventional optical imaging techniques which detect reflected or transmitted light, since ultrasound scattering is two to three orders of magnitude weaker than optical scattering in biological tissue [1]. Furthermore, PAI can provide not only anatomical, but also tissue characteristic and functional information by using optical property of tissue which can be extracted from PA signals. Taking advantage of these merits, medical applications were reported for example, functional imaging of brain [2] and high resolution imaging of superficial vascular [3].

Our goal is clinical application of PAI. In order to find the appropriate target to image, limitations of penetration depth and image resolution should be verified. In PAI, optical scattering and acoustic attenuation limits penetration depth. In this study, we experimentally evaluated penetration depth and resolution.

2. Material and Method

In order to evaluate penetration depth of PAI, we experimentally verified the depth of detection limit. In this experiment, phantoms placed in optical scattering material were imaged. We also evaluated the depth dependence of image resolution.

A schematic diagram of PAI system is shown in Fig.1. Second harmonic generation of Q-switched Nd;YAG laser (Minilite II, Continuum) with wavelength of 532 nm was used as excitation laser pulse. Repetition rate and full width half maximum (FWHM) of laser pulse were 10 Hz and 3-4ns, respectively. The laser was focused onto an end of optical fiber and then, guided to phantoms.

Our original acoustic sensor made of piezo-electric copolymer film P(VDF-TrFE) detected ultrasound. The size of rectangular shaped sensor element was 0.5 × 6.0 mm^2. P(VDF-TrFE) has relatively wide frequency band comparing to piezo-electric ceramic PZT which is widely used for ultrasonography probe [4]. Since P(VDF-TrFE) sensed most of frequency components of ultrasound induced by PA effect, information regarding to generation and propagation of ultrasound could be extracted from waveform. FET amplifier (SA-220F5, NF Electronic Instruments, 46dB, 0.1 - 80 MHz) amplified PA signal detected by acoustic sensor then, the oscilloscope measured the amplified signal. In order to obtain PA image, acoustic signals were measured at multiple positions by scanning acoustic sensor in direction horizontal to surface of sensor element using motorized stage.

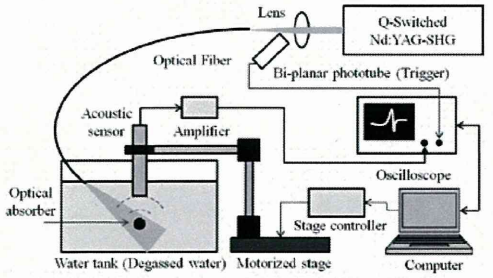

Fig. 1 Schematic diagram of experimental apparatus

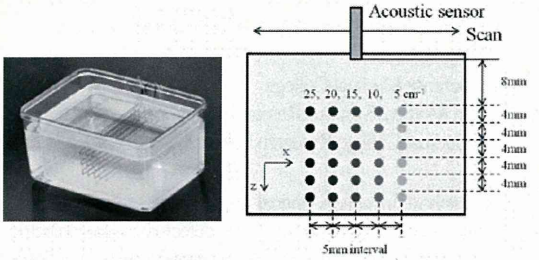

Fig.2 Arteriolar phantoms and schematic of its profile

Arteriolar phantoms made of silicon tube (inner diameter 0.5mm, outer diameter 0.6mm) filled with diluted black ink were imaged in this experiment. Optical absorption coefficient μ_a of phantoms could be adjusted by density of ink. Phantoms with different absorption coefficients were placed in arrangement shown in Fig.2. By imaging phantoms shown in Fig.2, the depth of detection limit could be clarified. The phantoms were placed in mixed medium of degassed water and optical scatterer (Intralipos). Scattering coefficient μ_s of ambient medium was adjusted by density of optical scatterer [5]. Since usage of near-infrared (NIR) light with deep penetration in biological tissue was preferred for clinical application, the reduced scattering coefficient μ_s' is adjusted to the value of muscle for NIR light [6].

In this experiment, the energy of laser pulse at the output of an optical fiber was adjusted to 6 mJ/pulse. The energy density less than damage threshold of biological tissue (ANSI safety limit: 20 mJ/cm^2) could be achieved by expanding diameter of laser beam. PA signals were measured at multiple measurement positions by scanning acoustic sensor 200 times with step of 0.2 mm. In order to enhance the signal to noise ratio, the signals were averaged 4 times. Image reconstruction algorithm was applied for signals measured at 200 positions to form a tomography.

3. Results and Discussion

As a result of experiment, the phantom with μ_a of 25 cm^{-1} was detected at a depth of 28 mm. However, image intensity decreased with μ_a. In case of μ_a =5 cm^{-1}, the intensity decreased to noise level at a depth of 20 mm. Although the absorption coefficient of blood in NIR depended on wavelength of laser and oxygen saturation, it was approximately 5-10 cm^{-1}. Therefore, the blood vessel at depth of 20-24mm could be imaged in this condition. Increase of laser pulse energy or use of optical contrast agents can increase the penetration depth.

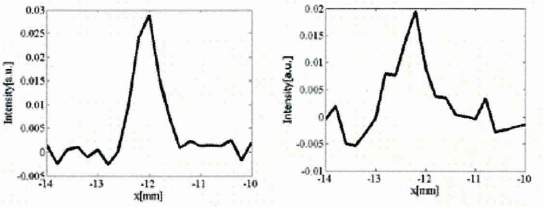

Fig.3 Lateral profile of photo-acoustic image of phantom with μ_a of 25cm^{-1} (Left : z = 12 mm, Right : z = 20 mm)

In order to evaluate resolution of PA image, full width half maximum (FWHM) in lateral direction of PA images were measured. Lateral profile of PA image of phantoms with μ_a of 25cm^{-1} placed at depth of 12 mm and 20 mm were shown in Fig.3. As shown in Fig.3, lateral FWHM 0.57 mm was obtained at depth of 12 mm and it increased at deeper region. Since this resolution corresponded to diameter of arteriolar, this method had resolution enough to image the vessels with larger diameter than arteriolar.

4. Conclusion

In this research, penetration depth and resolution of PAI were verified. They were important factors to decide the target to image. As results, blood vessel placed at 20-24 mm was detected in an ambient medium with scattering coefficient equivalent to biological tissue. Increase of laser pulse energy enables deeper tissue imaging. Furthermore, resolution enough to image arteriolar was confirmed. As next step, we discuss the appropriate target to image in clinical application.

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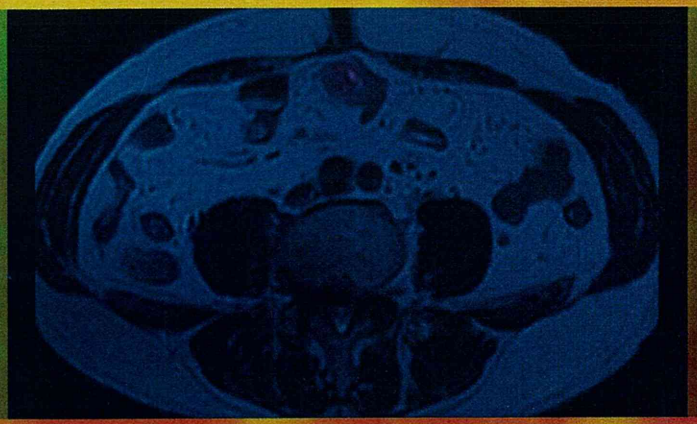

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