Phys. Med. Biol. **47** (2002) 1535–1541 PII: S0031-9155(02)32685-X

In vivo sonoluminescence imaging with the assistance of FCLA

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Received 14 January 2002
Published 19 April 2002
Online at stacks.iop.org/PMB/47/1535

Abstract
In this paper, sonoluminescence of tissues is reported for the first time. A sonoluminescence image of a living animal was obtained with a high-sensitivity imaging system. Furthermore, to enhance biological sonoluminescence, fluoresceinyl *Cypridina* luminescent analogue (FCLA), a chemiluminescence analysis agent, was used in the experiment. With the assistance of FCLA we succeeded in observing an image of a living animal with high contrast and good signal-to-noise ratio. This technique has potential applications in clinical diagnosis.

1. Introduction

Being non-invasive, non-ionizing, and with possible functional contrast for biomedical diagnosis, optical imaging of tissues has become an active research field in recent years (Alfano and Fujimoto 1996, Wang and Shen 1998, Yao *et al* 2001). Current fluorescence imaging techniques have the defects of autofluorescence disturbance from tissues and poor penetration ability for the short wavelength excitation laser. Because most biological tissues consist of water, ultrasound, as a mechanical wave, has excellent penetration ability and can be focused on any part inside the body, so it is an appropriate excitation source for clinics.

During the past ten years, much attention (Crum 1994, Hilgenfeldt *et al* 1999, Moss *et al* 1997) has been paid to the study of sonoluminescence (SL), which was discovered by Frenzel and Schultes (1934). SL arises from the cavitation process, in which bubbles filled with gas and vapour are generated within the liquid under ultrasonic action. Hilgenfeldt *et al* (1999) considered that SL was generated by thermal bremsstrahlung and recombination radiation

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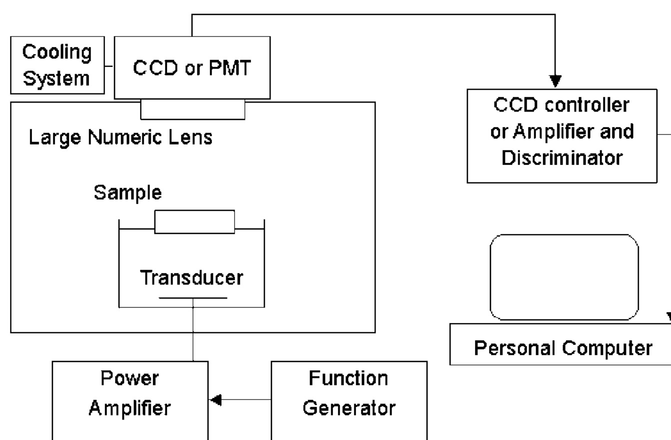

Figure 1. A schematic diagram of the experimental setup.

when bubbles collapsed, where the thermal bremsstrahlung is the inverse process of free–free transitions of electrons near ions and electrons near neutral atoms. In 1998, Wang and Shen (1998) first reported a technique of sonoluminescent tomography in tissue-simulating turbid media and predicted its potential applications in biomedicine. In their experiments, luminol was used to enhance SL, which is a lipid-soluble agent that works in alkalinity (pH 10) and can barely emit photons in physiological environments.

In this paper, we report experiments on SL imaging of a living body (nude mouse) and found that fluoresceinyl *Cypridina* luminescent analogue (FCLA), a chemiluminescent agent, can efficiently enhance SL by a reaction with oxygen free radicals (Nakano *et al* 1986) and work *in vivo*. Making use of a high-sensitivity charge coupled device (CCD) detector, the FCLA-enhanced SL image of a living body was obtained.

2. Experimental materials and methods

The experimental setup is shown in figure 1. The core part of it is the back-illuminated cooled CCD (512×512 pixels, Princeton Ins., TE/CCD-512TKB) detector where the pixel size of the camera is 22 μm and the spectral response range is from 300 to 800 nm with the peak around 500 nm. The average quantum efficiency is 60%. In order to reduce the thermal noise, which was down to 0.003 e/pixel s in our experiment, the CCD surface is cooled to -60 °C. The ST-130 controller dominates signal acquisition and transfers the data to the computer. In the tissue SL experiments, fresh porcine tissues (muscle, liver, fat) were cut into slices of $20 \times 20 \times 10$ mm for measurements. The SL light was detected by a photomultiplier tube (PMT). The PMT (CR-129, Hamamatsu) is biased at -1100 V and cooled to 0 °C to reduce the thermal noise. The signal from the PMT is amplified and discriminated into TTL pulses. The pulses are counted by the LabVIEW software (National Instrument) through an I/O counting card. The SL intensity was represented as counts per second (cps).

An ultrasonic transducer (Meza Guangzhou, China) which is 5 cm in diameter transmitted vertically an ultrasonic wave into the coupling medium. The ultrasonic transducer was driven by an amplified 40 kHz sinusoidal signal from a function generator (Tektronics, AFG320). The amplification was achieved using a power amplifier (ENI Co. Ltd, 2100L). The measurements with a hydrophone (HPM1/1, precision acoustics) showed that the ultrasonic pressure at the location of the mouse was proportional to the peak-to-peak voltage applied to the transducer.

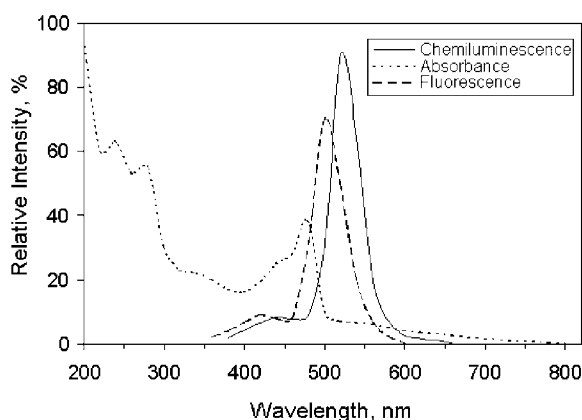

Figure 2. The spectra of absorption, fluorescence and chemiluminescence for FCLA (the excitation wavelength for fluorescence measurement is 283 nm).

The frequency of the ultrasound is 40 kHz. When the peak-to-peak voltage is 50 V, the peak ultrasonic pressure at the location of the mouse is 2.0 bars.

FCLA was dissolved in distilled water with a concentration of 5 μmol l^{-1} for *in vivo* experiments. The absorption and fluorescence spectra of FCLA solution were measured by a spectrophotometer (Hitachi, U-3400) and a spectrofluorophotometer (Shimadzu, RF-540), respectively. To measure the chemiluminescence spectrum of FCLA, a typical reaction system, OH^- + NaOCl + H_2O_2 (Murphy and Sies 1990), was used for singlet oxygen generation in which 1ml NaOH (5 mmol l^{-1}), 0.2 ml NaOCl (28 mmol l^{-1}) and 0.6 ml H_2O_2 (10 mmol l^{-1}) were mixed together. One millilitre FCLA solution (0.1 mmol l^{-1}) was added just before measurement.

Four week old male nude mice (BALB/c-nu/nu), with body length 6–8 cm and body weight 20–25 g, were used to establish a transplanted human glioma tumour. The weight and volume of the tumour were approximately 1 g and 1000 mm^3, respectively. The tumour-bearing nude mouse was anaesthetized with pentobarbital sodium (50 mg kg^{-1} ip) and then fixed on a metal mesh. FCLA solution (0.5 ml) was injected subcutaneously into the mouse 5 min before measurement. An appropriate amount of degassed water was poured into the pool to soak the abdomen of the mouse. The image acquisition time was 15 min. The water temperature was maintained at 15 °C by circulation. All the mice survived the experiments.

3. Results and discussions

FCLA was exploited recently as a highly efficient chemiluminescence analysis reagent. It can efficiently react with singlet oxygen $(^1O_2)$ or superoxide anion (O_2^-) to emit luminescence through a dioxytane intermediate (Nakano *et al* 1986). The spectra of absorption, fluorescence and chemiluminescence for FCLA were illustrated in figure 2, respectively. The absorption spectrum shows that the main region is below 300 nm and a weaker absorption peak around 490 nm. There is a peak around 510 nm for the fluorescence spectra of FCLA with the excitation wavelength being in the range of 244–491 nm. For the chemical reaction of FCLA with $^1O_2$ there exists a strong emission at 532 nm with a bandwidth of 40 nm (FWHM). The efficiency of the emission reaches a maximum at pH 7.5, which is just the physiological pH

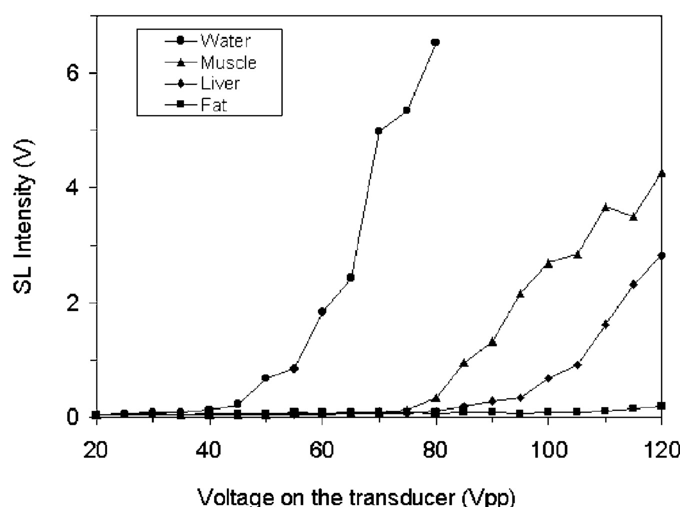

Figure 3. Effect of the driving voltage on the ultrasonic transducer on the SL intensity from the tissue-simulating media and porcine muscle, liver and fat tissues.

environment. Unlike other chemiluminescence reagents (e.g. luminol), FCLA is water-soluble and can be used conveniently *in vivo* (Skatchkov *et al* 1998).

We measured the relation between the SL intensity and the peak–peak voltage applied to the ultrasonic transducer in the tissue-simulating media and the porcine muscle, liver and fat tissues (figure 3). The ultrasonic pressure was proportional to the peak–peak voltage applied to the transducer. Because of the limitation of power on the ultrasonic transducer, we could not measure the SL in fat tissue with the present acoustic pressure. The result clearly showed that there were thresholds of ultrasonic pressure to generate SL in the muscle, liver tissues and the media. The thresholds are in the order: fat > liver > muscle > scattering media. Because of the limitation of power on the ultrasonic transducer, we could not measure the upper ultrasonic thresholds, although there must have been some. When the ultrasonic pressure increased above the threshold, the SL intensity increased rapidly with the pressure. The SL intensity of tissue is lower than that of the media when the same pressure is applied. The rapid increase of SL intensity with the acoustic pressure above the thresholds indicated that the SL signal would be a sensitive measure of tissue imaging.

Figure 4(a) is the photograph of a tumour-bearing nude mouse taken with dim light. The SL image of the mouse under the action of ultrasound is shown as figure 4(b). The purpose of using tumour-bearing nude mice in our experiments is to find out whether there is any difference of SL between tumour and non-tumour tissues. The location of the tumour was at the right side of the neck of the mouse. From figure 4(b), the shape of the mouse can be seen clearly, but no abnormality of the emission can be observed in tumour tissues. The emission intensity at every point in the mouse body is almost equal. Photon counting shows that the average intensity is about 4550 photons cm^{-2} s^{-1}, which is much lower than that of SL *in vitro* (can be seen by the eyes directly, Crum 1994). Considering the penetrability of light in the tissues, the SL of the mouse mainly comes from the superficial-layer tissues.

Figure 5(a) is the photograph of a nude mouse. 0.5 ml of FCLA solution had been injected subcutaneously at the position of the cross 5 min before measurement. Figure 5(b) is the image of the mouse under the action of ultrasound. Obviously, the emission around the cross is much stronger than that at other places. The emission intensity around the cross

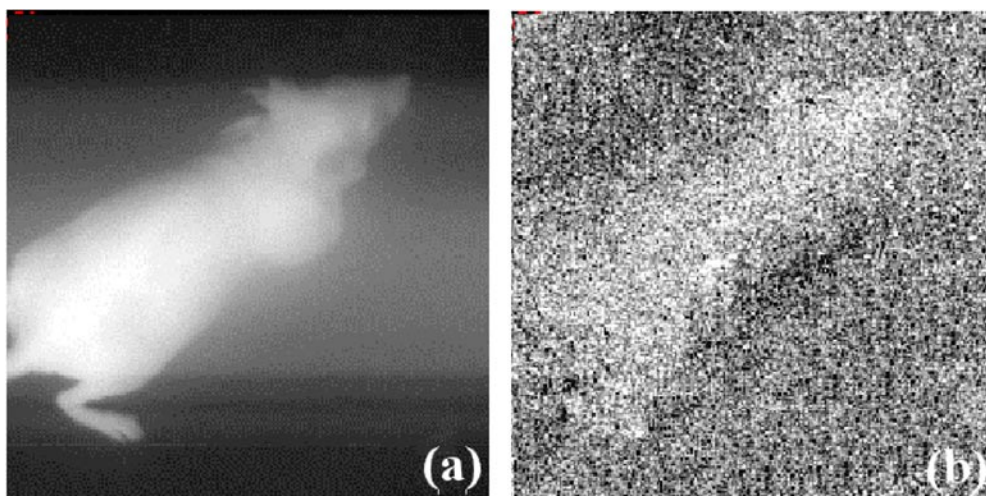

Figure 4. (a) The photograph of a nude mouse taken under dim white light, and (b) the SL image of the same mouse. The exposure time of (b) is 15 min. The mouse was alive during the imaging process.

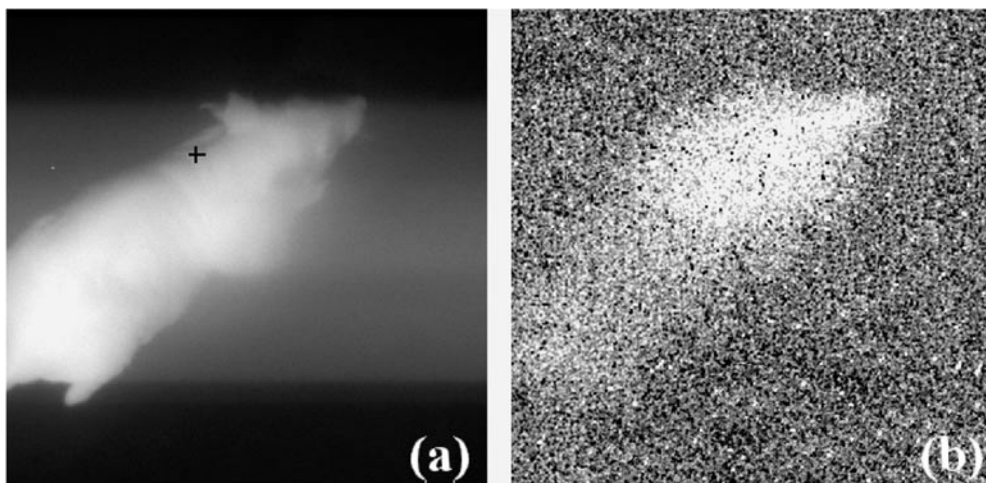

Figure 5. (a) The photograph of a nude mouse taken under dim white light, and (b) the FCLA-enhanced SL image of the same mouse. FCLA solution was injected subcutaneously at the cross, and the exposure time of (b) is 15 min. The mouse was alive during the imaging process.

reaches 12 580 photons cm^{-2} s^{-1}, while being only 4360 photons cm^{-2} s^{-1} at other regions. We attribute the enhancement around the cross to the FCLA-enhanced SL, because FCLA must have diffused into the tissues around the cross within several minutes after injection into the mouse. The strong emission at the cross is believed to result from the reaction of FCLA with $^1O_2$ or $O_2^{\bullet-}$ generated in ultrasound cavitation, while the weak emission at places away from the cross is the biological SL as in figure 4(b). In the same experimental arrangement without ultrasound irradiation, the photon emission intensity of either the FCLA region (caused by FCLA chemiluminescence) or non-FCLA region (by ultra-weak bioluminescence, which has

an intensity of several tens of photons cm^{-2} s^{-1}) is too low to be detected from the noise background.

The spectrum of SL generated in different liquids is distinct (Crum 1994). In the case of water, there is a well-defined peak at 310 nm, which is associated with the molecular bands of the $OH^{\bullet}$ free radical that is produced by the high temperature and pressure within the bubbles. It is known that 70% of an animal body is water and oxygen is transported continuously to every point of the body through the respiratory and the circulatory systems. When ultrasound cavitation occurs in tissues, the high temperature and pressure within the bubbles will provide an appropriate environment for chemical reactions to produce oxygen free radicals. So, the biological SL in figure 4(b) probably results from the direct de-excitation and the reaction with chromophoric biomolecules of the free radicals (Murphy and Sies 1990). If FCLA is injected into the tissues, it will efficiently react with $^1O_2$ or $O_2^{\bullet -}$ and result in a strong chemiluminescence to enhance the biological SL (figure 5(b)). We found that the emission from dead mice is much weaker than that from living mice. This phenomenon might result from the cessation of respiration, which makes it impossible to provide oxygen to tissues.

It is known that ultrasound has harmful effects on tissues through the processes of cavitation and free radical formation for acoustic pressure greater than a critical value. In our histological analysis experiments, the results show no differences between the groups with and without ultrasound treatment under the present ultrasound intensity. In the present experiments, the acoustic pressure is 2.0 bars, corresponding to a spatial-peak–temporal-peak power of 1.3 W, far less than the safety limit of 23 bars set by the US Food and Drug Administration (Shen and Wang 1999). Therefore there should be an ultrasonic safety window within which SL can be used to image tissue without causing tissue damage.

We think the biological SL observed in the present experiments mainly comes from the superficial tissues because of the absorption by tissues. However, if we select another chemiluminescence reagent that has similar properties to FCLA and can emit longer wavelength photons (680–950 nm), and utilize the confocal scanning technique (Xing *et al* 1999), it is possible to achieve deep-layer tissue tomography. This method could be further combined with endoscopy to become a practical technique in clinical diagnosis. SL-enhancement of tumour tissues was not observed in the present experiment. If a sonosensitizer (e.g. ATX-70, Kessel *et al* 1994), which can produce singlet oxygen after sonosensitization (Yumita *et al* 1989) by ultrasound, is used to localize the cancer tissues, least-invasive tumour diagnosis in clinics could be possible, especially for use in the detection of breast cancer. Since breast cancer tissue is relatively thin, SL light emission might be measured in women *in vivo*, and the fat tissue would not interfere with the SL signal.

4. Conclusions

In conclusion, SL of several types of tissue was measured, and a living body was imaged using tissue's SL. FCLA is an excellent chemical agent that can enhance the SL efficiently and can work *in vivo*. We also observed the FCLA-enhanced SL image of a living body with a high-sensitivity imaging system. With confocal techniques, this imaging method has potential applications in clinical diagnosis and can be used for tumour diagnosis with sonosensitizers being used to localize the cancer tissues.

Acknowledgments

This work was supported by the National Science Foundation for Distinguished Young Scholars of China (no 69725009) and the Team Project of Natural Science Foundation of

Guangdong Province (no 015012), and acknowledgments are given to Dr Xiangbo Yang and Dr Xueyun Zhong.

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