Chapter 3

Development of LD 3 Wavelength Pulsed Laser for PDD and PDT

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Abstract: A new LD (Laser Diode) was developed to specifically excite protoporphyrin IX (PpIX), a photochemotherapeutic agent that is selectively produced in human tumor tissues following administration of 5-aminolevulinic acid (5-ALA), as well as its photoproduct. PpIX emits red fluorescence if it is exposed by the blue light (410 nm) of the laser. During fluorescent diagnosis, the 2 wavelengths of red excitation (635 and 665 nm) from the LD laser were both exposed to the tumor at the same time. Both

wavelengths were effectively absorbance matched for the PpIX and the photoproduct produced during irradiation. There is an overlap in the spectral characteristics of the PpIX absorbance and emission. Both processes occur at 635 nm. As such, a continuous wave laser cannot excite PpIX and monitor its emission simultaneously. We therefore developed a pulsed CW laser that could time gate between the blue and red lights, enabling us to both excite and monitor the PpIX at the same time. In future, this laser will be used in a risk assessment protocol for the treatment of elderly patients and to also assess the relative cost this treatment in comparison with other cancer treatments.

Introduction:

5-aminolevulinic acid (5-ALA) is a precursor of heme, synthesized from succinyl coenzyme A and glycine. It contributes to a chain of porphyrin intermediates (porphobilinogen, uroporphyrinogen and coproporphyrinogen) leading to mitochondrial generation of protoporphyrin IX (PpIX) using 8 molecules of 5-ALA. The latter is then enzymatically converted to heme via ferrochelatase. These reactions briskly proceed in the liver and in erythrocyte precursors even in healthy individual but are augmented in rapidly proliferating cells. The usage of 5-ALA dosing, metabolism typically halts at porphobilinogen (PBG) due to rate-limiting PBG deaminase activity. However, the rate-limiting step in more active tumor cells shifts to ferrochelatase, so that Pp-IX accumulates instead and it is the accumulation of PpIX that responds to ultraviolet light, emitting a red fluorescence. Furthermore, we observed that PpIX fluorescence was enhanced by adding a NO generator, S-nitroso-N-acetylpenicillamine (SNAP), whereby the NO radical blocks the ferrochelatase enzyme activity.

First generation laser systems, such as eximer-dye lasers, an OPO-lasers and argon - dye lasers have been used as excitation sources in clinical photodynamic therapy (PDT) during the past 20 years . The high purchase and maintenance cost of these older lasers have become problematic for clinical applications. Laser diodes (LD), such as the one emitting at 664 nm (Panasonic, Ltd. Co., Tokyo) shows some promise in PDT, but is limited to a single wavelength.

During ALA-PDT, the absorption of the photosensitizer in the tumor tissue will actually undergo a change in absorbance characteristics during the irradiation period. Ideally, the wavelength(s) produced by the excitation laser should also be capable of exciting the modified photosensitizer. The two principle excitation wavelengths for PpIX are 410 nm (Soret band) and 635 nm (Q-band). In addition, it is now known that the PpIX photoproduct, produced during the initial phase of PpIX photoexcitation either at 410 or 635 nm, has an absorbance peak at 665 nm (3). A further complication is that

635 nm is both at an absorption and emission peak of PpIX   Thus, one would like to be able to separate the absorbance process from the emission process, especially during the photodetection/photodiagnostic (PD) phase.   The new LD laser used in this study has three excitation wavelengths, 410 nm, 635nm and 665 nm, corresponding to the main absorbance peaks of PpIX as well as the absorbance peak of the PpIX photoproduct. Further, in order to separate the absorbance process from the fluorescence process, one can time gate (pulsed timing delay) the laser so as to separate the absorbance phenomenon from the fluorescence process. The 3 wavelengths LD-laser was pulsed for the timing of the fluorescent diagnosis and the PDT treatment in order to remove the fluorescence and irradiation wavelength overlapped at the same wavelength (635 nm). One can thus separate the red fluorescent emission and the PDT irradiation at 635 nm through the pulsed trimming delay.

Experimental and Methods:
(1) Photoproduct during PDT irradiation.

The excitation spectra   of all absorbing species in 10^5 cells/ml HL-60 cell (human leukemia) suspension incorporated 0.1 mM 5-ALA for 30 min incubation   was obtained by monitoring at the 635 nm fluorescent peak and changing the excitation wavelength using a fluorescence spectrophotometer (Type-850, Hitachi Ltd., Co., Tokyo) as shown in Figure 1. The excitation spectrum of (A) was in the case of before irradiation and (B) was after irradiation (40 J/cm^2) at 630 nm, respectively.   The (B) spectrum was changed at the Q-band of the porphyrin, specifically showing a decrease of the excitation peak intensity at 635 nm with a corresponding increase at 665 nm. The new peak of 665 nm is probably a chrorin-E6 type of PpIX derivative, as shown in the Diels-Alder reaction in Figure 1.
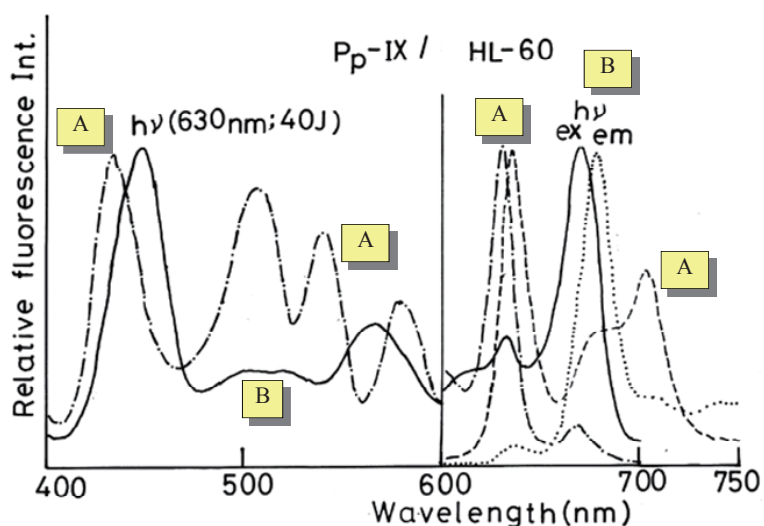
[Figure 1] The excited fluorescent spectra of Pp-IX and the photoproduct
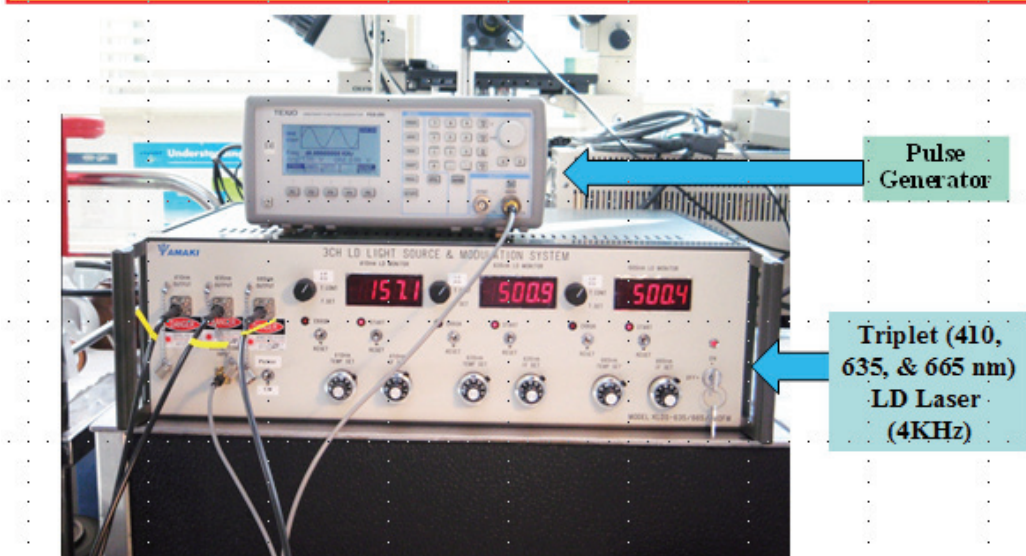
produced before (A spectrum) and during (B spectrum) irradiation at 630 nm (40 J/cm^2) against the Pp-IX molecules incorporated in the HL-60 cultivated cell suspension (The upper chemical reaction of Diels-Alder type) is speculated to produce the isomer molecules of Chorine E6 derivatives.

In actual, the excitation peak at 630 nm was decreasing with increasing the excitation peak at 665 nm during the photo-irradiation. As it has been reported about the photoproducts of Pp-IX by many researchers, chlorine derivative by Moan group (4, 5, 6), we can also estimate that it will be a chlorine-like derivative from the matting with the excitation (665 nm) and emission (670 nm) peaks of the derivative. Furthermore, the product plays photoactive as a photosensitizer as following figures. On the other hand, it was reported that the photoproduct will a hydroxyl aldehyde derivative of Pp-IX reported by Dietel, et al. (7) as the product of Pp-IX.

Results

The prototype of 3 wavelength pulsed LD-laser is shown at Figure 2, which includes a pulse generator for the three wavelengths (410, 635 and 665 nm) LD laser at 10 kHz.   The maximum output power for these three wavelengths was more than 150 mW/cm^2 at 4 kHz. The time-dependent profile for these wavelength output are shown in Figure 3.   The delay time between pulses was 38 μsec for both 635 and 665 nm).
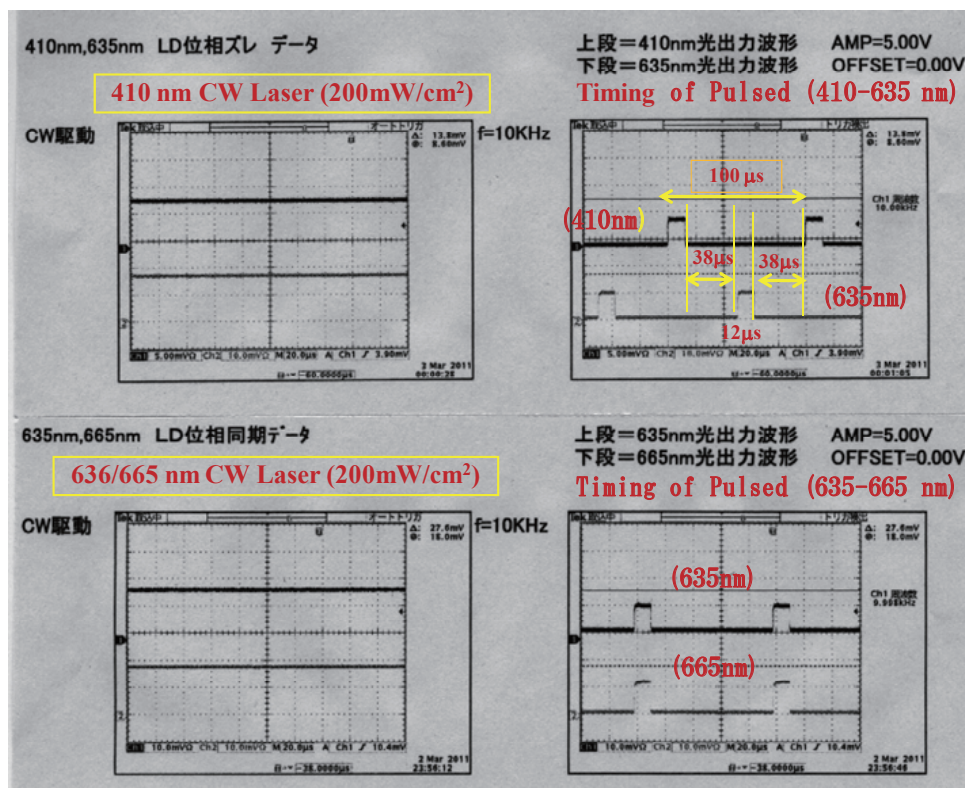
[Figure 3] The shape of timing control of the excitation and irradiation pulses. (Left side)=CW laser, (Right side)= Pulsed timing of excitation at 410nm and irradiation at 635 and 665 nm. The lag time between the excitation and the irradiation is 38 μsec at 10 kHz. Each pulses are 12 μsec at 10 kHz.

In living animal experiments, both of the excitation and treatment irradiation were done successfully as shown in each photograph, excitation and treatment irradiation of experimental tumor model nude mouse, which were explanted with human prostate cancer (grade-IV) cell line (PC-3: with cell density 1 x 10^6 cells) under the skin (subcutaneous injection) of the femur at 4 hours after the oral administration of 1 mM 5-ALA saline solution. The fluorescent red emission was observed by the excitation of LD laser at 410 nm and at 4 kHz pulsed. The irradiation of 2 wavelength for 4 kHz pulsed laser light for PDT was confirmed as the right photograph in Figure 4 by a time-

dependent camera (Streak Camera Type, Hamamatsu Photonics, Ltd. Co. Hamamatsu). The distribution of the red fluorescent image was a close match to the part of tumor tissue in the left photograph. Furthermore, the tumor region was focused by the irradiated red-lights of 635 and 665 nm independent of the fluorescent region. The dose of treatment was 150 J/cm^2 for changing the color of dark red and edema in all areas of tumor tissue at 12 hr after the irradiation.

Discussion

The photos shown in Figure 4, clearly show the ability of the three wavelength LD laser to monitor the PDT treatment via its fluorescence diagnosis simultaneously while excitation is taking place, as observed on the monitor. This novel laser system can thus be very useful for both the treatment and photodiagnosis of PDT.   It is our hope that this new system will provide an easy to use system capable of producing reproducible results for the monitoring of tumor tissues of various types at different stages of the PDT treatment. Furthermore, the survival rate of the tumor model mice can be assessed with such a system.

Photograph for PD (410 nm) and PDT of Pp-IX (635 nm) and the Photoproduct (665 nm)
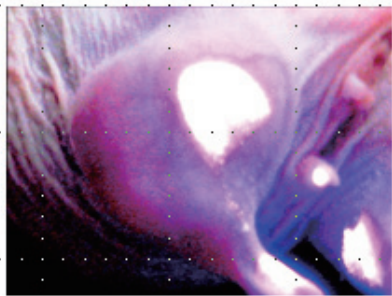
Fluorescence detection by LD Laser (410 nm) Timing (4 kHz)
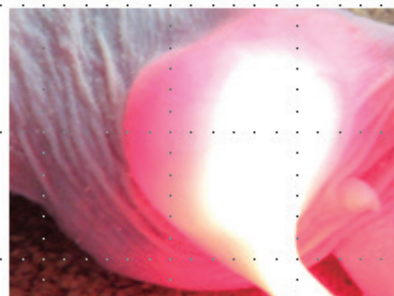
Irradiation of 2 wavelength (PDT) for 4 kHz Against a transplanted Tumor (Prostate cancer) Model

[Figure 4] Photograph of fluorescent photo detection (PD) image and irradiated image of the tumor tissue (a human prostate cancer model nude mouse) transplanted the cell line (PC-3).
These images were observed by a streak camera (C-5680 type; Hamamatsu Ltd. Co, Hamamatsu.) for each different   pulses.

Acknowledgments

References

1. Miyoshi, N., Fukunaga, Y., Kaneko, S., and Hisazumi, H. PD ad PDT with porphyrin precursors (5-ALA) in Japan. *J. Jpn. Soc. Laser Surg. and Med.*, **29 (2):** 164-169 (2008).

2. Miyoshi, N., Ogasawara, T., Nakano, K., Tachihara, R., Kaneko, S., Sano, K., Fukuda, M., and Hisazumi, H. An application of fluorescence analysis in tumor tissue. "Recent Progress on Clinical and Basic Research of ALA" Edited by Miyoshi, N., and Kaneko, S., Published by Kashiwaba Neurosurgery Hospital, June (2005).

3. Konig, K, Schneckenburger, H., Ruck, A., and Steiner, R. *In vivo* photoproduct formation during PDT with ALA-induced endogeneous porphyrins. *J. Photochem. Photobiol. B. (Biol)*, **18**, 287-290 (1993).

4. Baqdonas S, Ma LW, Lari V, Rotomskis R, Juzeras P, Moan J. Phototransformations of 5-aminolevulinic acid-induced protoporphyrin IX in vitro: a spectroscopic study. *Photochem. Photobiol.*, **72 (2):** 186-192 (2000).

5. Ma L, Baqdonas S, Moan J. The photosensitizing effect of the photoproduct of protoporphyrin IX. *J. Photochem. Photobiol. B*, **60 (2-3):** 108-1013 (2001).

6. Juzenas P, Lani Y, Baqdonas S, Rotomskis R, Moan J. Fluorescence spectroscopy of normal mouse skin exposed to 5-aminolaevulinic acid and red light. J. Photochem. Photobiol. B, 61 (1-2): 78-86 (2001).

7. Dietel W, Fritsch C, Pottier RH, Werndenburg R. 5-aminolaevulinic-acid-induced formation of different porphyrins and their photomodifications. *Lasers Med. Sci.,* **12 (3):** 226-236 (1997).