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Photobleaching behavior of protoporphyrin IX during 5-aminolevulinic acid marked glioblastoma detection

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ABSTRACT

The highly malignant brain tumor, glioblastoma multiforme (GBM), is difficult to fully delineate during surgical resection due to its infiltrative ingrowth and morphological similarities to surrounding functioning brain tissue. Selectiveness of GBM to 5-aminolevulinic acid (5-ALA) induced protoporphyrin IX (PpIX) is reported by other researchers to visualize tumor margins under blue light microscopy. To allow objective detection of GBM, a compact and portable fiber optic based fluorescence spectroscopy system is developed. This system is able to deliver excitation laser light (405 nm) in both the continuous and pulsed mode. PpIX fluorescence peaks are detected at 635 and 704 nm, using a fiber-coupled spectrometer. It is necessary to optimize the detection efficiency of the system as the PpIX quickly photobleaches during the laser illumination. A light dose of 2.5 mJ (fluence rate = 9 mJ/mm^2) is experimentally approved to excite an acceptable level of fluourescence signal arising from glioblastoma. In pulsed illumination mode, an excitation dose of 2.5 mJ, with a dark interval of 0.5 s (duty cycle 50%) shows a significantly shorter photobleaching time in comparison to the continuous illumination mode with the same laser power ($p < 0.05$). To avoid photobleaching (the remaining signal is more than 90% of its initial value) when measuring with 2.5 mJ delivered energy, the time for continuous and pulsed illumination should be restricted to 2.5 and 1.1 s, respectively.

Keywords: Photobleaching, glioblastoma multiforme, 5-aminolevulinic acid induced protoporphyrin IX, fiber optic based fluorescence spectroscopy

1 INTRODUCTION

The highly malignant brain tumor, glioblastoma multiforme, is difficult to fully resect due to its infiltrative way of growing and its morphological similarities to surrounding functioning brain under direct vision in the operating field. Magnetic Resonance and Computed Tomography images are taken before and after surgery for observing the suspected tumor tissue but still the important task of identifying tumor margins is based on intraoperative visual inspection and palpation of tissue. The importance of complete tumor resection in survival rate of patients is studied previously [1].

Optical measurement is a precise and spatially beneficial option for assisting the neurosurgeons with delineating the border between normal and tumor tissue during tumor resection. Alternatives of fluorescence imaging and measuring on endogenous [2, 3] and exogenous [4, 5] type of fluorophores have been reported. Although fluorescence image guided brain tumor resection is approved for clinical use in Europe, it is still far from a clinical consensus on how to best utilize fluorescence labeling and information in practice. Therefore, further research and development of clinically suitable fluorescence methods is essential.

The general idea in this study is to orally administer a low dose of 5-aminolevulinic acid (5-ALA) prior to surgery. This drug passes the blood-brain barrier in the tumor and is converted to the fluorescence marker protoporphyrin IX (PpIX) in the malignant cells, acting as a tumor marking substance. PpIX is a natural substance in the heme cycle which is rapidly eliminated from the body. Laser light at 405 nm is absorbed by protoporphyrin IX. The molecules of PpIX re-emit fluorescence with peaks at 635 nm and 704 nm. This light is used for tumor delineation.

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Accumulation of PpIX in the infiltrating cancerous cells of different tissues including brain has been reported [6]. A high specificity of PpIX accumulation has been reported in glioblastoma [4, 7]. The reason is mostly referred to the disrupted blood brain barrier in tumors [8] as well as altered levels of heme cycle enzymes, including porphobilinogen and ferrochelatase, in tumor tissue which hinder the transformation of PpIX into heme [6, 9, 10]. This makes them fluoresce when exposed to light of appropriate wavelength. Higher progression free survivals in resection guided 5-ALA induced fluorescence microscopy has been reported by Stummer et al. [4].

This study was conducted using a compact fiber optic based fluorescence spectroscopy system benefiting from a laser module of 405 nm wavelength (maximum power of 50 mW) with functionality in continuous and pulsed modes. The system is based on a pilot study previously conducted by Illias et al. [11]. The performance has been evaluated both on skin and during neurosurgical glioblastoma resection procedure; however, the neurosurgical application of the results was in focus of the study.

To achieve the optimal performance, it is of importance to adjust the laser energy delivered to the brain at balance between a proper signal to noise ratio and minimal photobleaching. Therefore, this work is aimed as a behavioral photobleaching study of 5-ALA induced PpIX in 5-ALA treated skin and glioblastoma during neurosurgical resection, in addition to the energy optimization of the excitation light.

2 MATERIALS AND METHODS

2.1 System

The different components of the system, as described below, are mounted in a compact box of 31×25×21 cm which is easily carried on a trolley to the operation theater. The laser light output power was measured prior to recording with a laser power meter (Ophir-Spiricon, Ophir Optronics Ltd., Israel), while the detection unit of the system was calibrated with well characterized light sources.

The excitation light is delivered by a violet laser diode module emitting at 405 nm with a maximum power of 50 mW (Oxxius SA, France). The laser functions in pulsed and continuous wave modes. A spectrometer (EPP 2000, Stellarnet, USA) with 2048 element CCD capturing light in the range of 248-850 nm is used for recording the laser-induced fluorescence emitted by the tissue. The spectral resolution of the recorded spectra is about 3 nm. A long pass filter (Schott, CVI, USA) eliminates the laser reflection light at 400 nm from entering the spectrometer.

A hand held fiber probe is used as the interface between tissue and the system. An optical fiber in this probe with core diameter of 600 um and numerical aperture of 0.37 brings the excitation light from laser to the tissue. This excitation fiber is surrounded by receiving fibers which collect the light from the measurement site and transfer it to the spectrometer. Three different probe configurations were used. In two probes, the receiving fibers were of 200 um core diameter and numerical aperture of 0.22. The third probe included two receiving fibers of 600 um and 0.37 numerical aperture. The optical fibers at the detector end were arranged to match the slit configuration of the spectrometer. All probes had a diameter of 3 mm. A fiber port (OFR Inc., Caldwell, NJ, USA) was used at the interface of the probe and laser for light alignment.

The system operates both in the continuous and pulsed modes and is controlled via a software interface (LabVIEW®, National Instruments Inc., USA) and a DAQ card (National Instruments Inc., USA). The system is chosen to operate at three different excitation light power densities: 3.6, 17.8 and 37.5 mW/mm^2 at the tissue surface. In continuous mode, the laser irradiates continuously at this power density while the spectrometer collects light. The integration time stands for the time interval within which the spectrometer collects tissue fluorescence. This time sets the intensity levels of the collected spectra. When the system is used in this mode, the lamp light reaching the system has to be manually suppressed. In pulsed mode, the laser pulses with a duty cycle of 0.5 and the spectrometer collects light synchronized with the laser light and dark pulses. Each dark spectrum is then subtracted from the light spectrum recorded immediately after to minimize the influences of the light present in the operation theater. In this case integration time of the spectrometer is set equal to the laser pulse width within the range of 0.2 to 2 s.

2.2 Measurements

Measurements were performed in two phases on skin and glioblastoma. The administration method of 5-ALA has been different on skin compared to brain as explained below under each category. In both cases the probe has been held stationary at one point in contact with the tissue while the fluorescence spectra was recorded.

2.2.1 Measurements on skin

Metylaminolevulinat (METVIX® 160 mg/g, Photocure ASA, Norway) cream was topically applied in a 2-3 mm thick layer over an area of 3 cm^2 on the tape stripped skin of forearm 4 hours prior to the measurements. The aminolevulinic acid of the cream converts to protoporphyrin IX in the skin through the heme cycle of the cells (the highest PpIX signal is found first after at least 3 hours according to our experience). The skin was meanwhile covered with a nonabsorbent dressing (Tegaderm™, 3M Health Care, Germany) and a nontransparent plaster to restrict photobleaching of the cream induced PpIX. Measurements were performed on one person on different days in both the continuous (n = 26) and pulsed (n = 63) modes. Pulsewidths of 0.2 to 1.5 s were used.

2.2.2 Measurements on glioblastoma

Patients undergoing surgical resection of glioblastoma (n = 4) were given a 5 mg/kg bodyweight of 5-ALA dissolved in orange juice 2 hours prior to skull opening. They were then anesthetized as a preparation for surgery. The fiber probes used were sterilized with the STERRAD® procedure. Measurements were approved by the local ethics committees (No: M139-07) and written informed consent was received from all the patients.

Each measurement procedure was restricted to a maximum of 3 minutes. As the photobleaching measurement time is relatively long, any possible exposure of healthy tissue was avoided and the measurements sites were confined to malignant tumor far from the tumor border (n = 9) or freshly removed tumor (n = 22). The surgeons, who were acquainted with the measurements, held the probe in a stable position for in vivo measurements. The fluorescence signal was then recorded in approximately 2 minutes until the signal was almost completely photobleached away. Light powers of to 1 and 5 mW and an integration time of 0.5 s (with an exception of 2 s in one continuous mode case) was used in the measurements. Measurements were performed in both the continuous (n = 18) and pulsed modes (n = 13).

2.3 Data analysis

The data were analyzed using MATLab 7.0 (The MathWorks™, Inc., USA). The tissue autofluorescence of each spectrum gives a reference, compensating for the different probes applied and the probe position parameter [12]. Thus, the ratio of the fluorescence peak at 635 nm to the autofluorescence peak, after reduction of the dark spectrum, both in the very first collected spectrum, was taken as a value for the initial intensity level evaluation. This value is referred to as the *ratio number* in the text.

The photobleaching has been evaluated by the time when the intensity reaches 90 %, 37 % and 10 % of its total value. These time coefficients ($t_{90\%}$, $t_{37\%}$, $t_{10\%}$) were obtained by subtracting the base level (when the PpIX is completely bleached but the tissue autofluorescence is above zero) from the first collected value where fluorescence is at its maximum and then multiplyed by the assigned percentage. In some measurements, the $t_{37\%}$ and/or $t_{10\%}$ were much longer than the probe could be held still. Thus, relatively long time coefficients were attributed to them.

One and two tail paired and unpaired t-test and ANOVA test (p value), depending on the nature of the comparison, has been applied to investigate the statistical difference between time coefficients in measurements with different laser powers, continuous and pulsed irradiation measurements and pulsed mode measurements with different pulsed doses. Statistical correlation coefficient, r, was used to find correlation between photobleaching time coefficients and the possible affecting factors. The p and r values which are mentioned just for an individual time coefficient were marked by the corresponding time coefficient index; for instance, p-value calculated for $t_{37\%}$ of a group of data is noted by $p_{t37\%}$ while p is obtained for all the three time coefficients. P-values of less than 0.05 were considered to stand for statistically significant differences.

3 RESULTS

Typical fluorescence spectra of non-treated and ALA treated skin, Fig. 1 (a), and malignant tissue compared to gray matter from a patient diagnosed with malignant glioblastoma, Fig. 1 (b), are presented. PpIX fluorescence peaks at 635

and 704 nm are apparent in the malignant tumor. Signals recorded from gray matter of the cortex show no PpIX peaks. The ratio number in the glioblastoma samples (12.1 ± 8.6, n = 31) is much higher than in the skin (0.6 ± 0.4, n = 89). Experimentally, a minimum of 2 mJ excitation energy gives an acceptable signal level when measuring in brain.

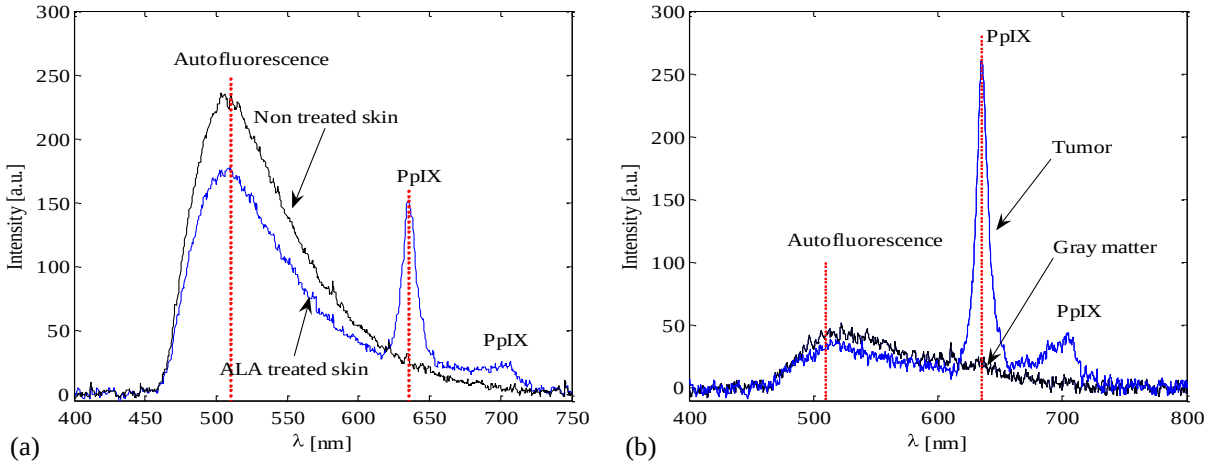

Fig. 1. Fluorescence signal recorded from (a) 5-ALA treated and non treated skin, and (b) a glioblasoma patient. 5 mW laser power with 0.4 s integration time (2 mJ excitation) was used. PpIX peaks are apparent at 635 and 704 nm. The dotted lines at 510 and 635 nm show the points where photobleaching was further analyzed.

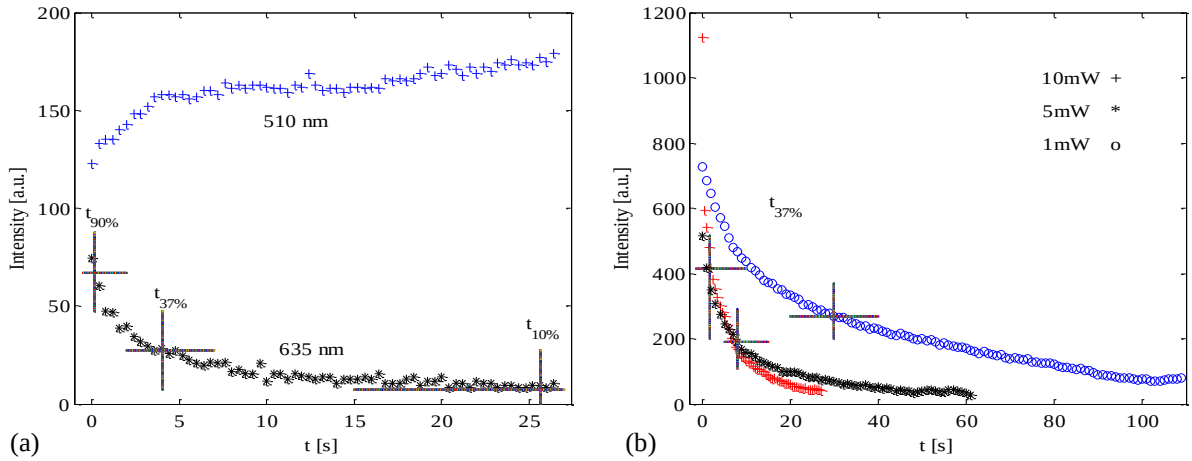

Fig. 2. Photobleaching in skin (a) at 510 and 635 nm under 5 mW laser power with 0.5 s integration time, and (b) Typical photobleaching at 635 nm peak as a function of laser power. The marks present the time when intensity reaches 37% of its total value ($t_{37\%}$). Tissue autofluorescence shows an incremental behavior at the start of the curve.

An example of change in fluorescence level in ALA treated skin at wavelengths of 510 nm (tissue autofluorescence) and 635 nm are illustrated in Fig. 2 (a). Photobleaching curves at 704 nm wavelength follow the same pattern in each respective measurement in both skin and brain and are thus excluded from further analysis. Fig. 2 (b) shows behavior of fluorescence change at 635 nm as a function of laser power. A photobleaching curve obtained from a fresh tumor sample is presented in Fig. 3 (a), and typical curves of PpIX photobleaching in brain with pulsed and continuous excitation lights (2.5 mJ) are presented in fig 3 (b). Two tail unpaired t-test on one patient does not show any significant difference between in vivo (n = 6) and ex vivo (n = 6) measurements.

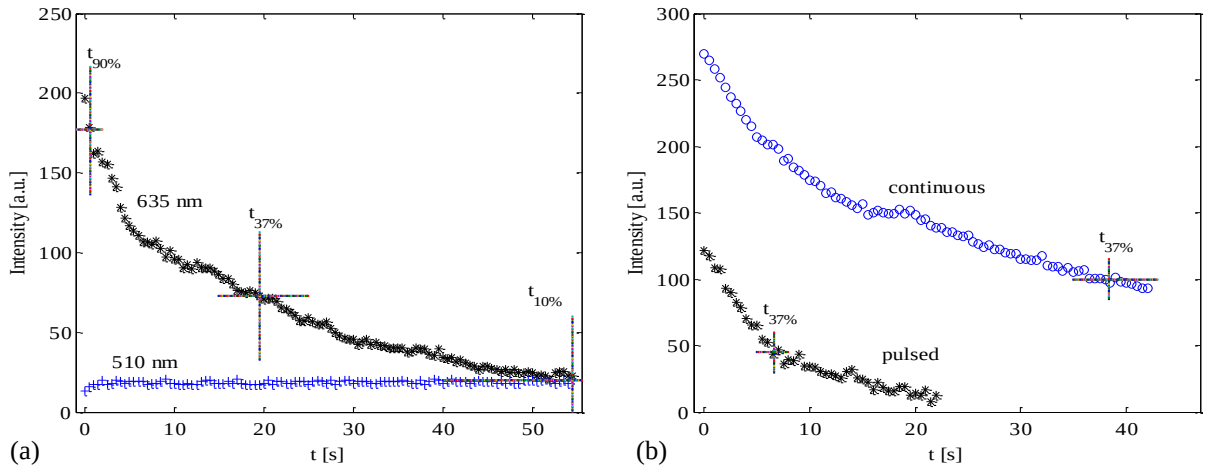

Fig. 3. Photobleaching in glioblastoma sample with laser power of 5mW and integration time of 0.5 s (2.5 mJ): (a) Change of PpIX fluorescence and tissue autofluorescence in a continuous mode measurement, and (b) Photobleaching of PpIX at 635 nm in a continuous and a pulsed mode measurement.

Summary of the photobleaching time coefficients measured in brain and skin is presented in Table 1. Pulsed measurements in average showed a faster photobleaching. In addition, measurements in brain demonstrated obviously slower photobleaching compared to skin. $t_{90\%}$ is considered as a limit before which minimal photobleaching happens. Derived from Table 1, this limit can be estimated for the desired excitation energy. Some correlation is seen between power and continuous measurements in skin and brain ($r_{37\%}$ = -0.6 and $r_{37\%}$ = -0.1).

Table. 1. Time coefficients of PpIX photobleaching (at 635 nm) in skin and brain measured in continuous and pulsed mode. Pulse width in pulsed measurements mentioned here is 0.5 s for both skin and brain. The data were rounded to the nearest decimal number.

TIME COEFFICIENTS OF PPIX PHOTOBLEACHING IN SKIN

Power	n	t (90%) m ± SD [s]	t (37%) m ± SD [s]	t (10%) m ± SD [s]
Continuous				
1 mW	7	0.3 ± 0.6	17.4 ± 11.7	48.5 ± 33.4
5 mW	11	0.3 ± 0.2	8.8 ± 5.4	42.6 ± 17.0
10 mW	8	0.2 ± 0.1	4.1 ± 2.0	19.8 ± 8.6
Pulsed				
1 mW	0			
5 mW	5	0.3 ± 0.3	4.8 ± 1.8	12.5 ± 3.5
10 mW	6	0.2 ± 0.1	2.3 ± 0.9	8.7 ± 2.9

TIME COEFFICIENTS OF PPIX PHOTOBLEACHING IN BRAIN

Power	n	t (90%) m ± SD [s]	t (37%) m ± SD [s]	t (10%) m ± SD [s]
Continuous				
1 mW	9	5.1 ± 7.6	36.5 ± 22.5	75.3 ± 31.3
5 mW	7	2.5 ± 2.2	42.2 ± 24.0	66.5 ± 16.8
10 mW	2	1.7 ± 0.0	26 ± 5.7	42.5 ± 3.5
Pulsed				
1 mW	1	1.6	7.4	50
5 mW	11	1.1 ± 1.1	20.2 ± 14.1	33.6 ± 14.7
10 mW	0			

In general, the tissue autofluorescence, both in skin and brain where ALA is administered, shows an increase in the few first spectra and stays fairly constant afterwards (Fig. 2 (a), 3 (a)). The standard deviation of the tissue autofluorescence of ALA treated skin is increased to (4.2 ± 3.5) % in all the continuous skin measurements. This variation in non ALA treated skin autofluorescence during 80 seconds of measurement was 2.5 % of the mean autofluorescence value (n = 1).

Fig. 4 shows the boxplots of the continuous measurements in skin as a function of laser power. Although not linear, the time coefficients decrease with the increased laser power. Fig. 5 shows boxplots of time coefficients of different pulse

widths compared to the corresponding continuous measurements with 5 and 10 mW laser power, all in skin. Using 5 mW, pulsated illumination makes a significant statistical difference only when pulses of 0.4 s intervals are used ($p_{t37\%} < 0.05$). When using 10 mW laser power, significant statistical difference appears only when 0.5 s pulsations are applied. The same comparison for glioblastoma measurements is illustrated in Fig. 6. Pulsed excitation imposes lower photobleaching time coefficients relative to the corresponding continuous illumination in brain when using 5 mW laser power with dark intervals of 0.5 s ($p_{t37\%} < 0.05$).

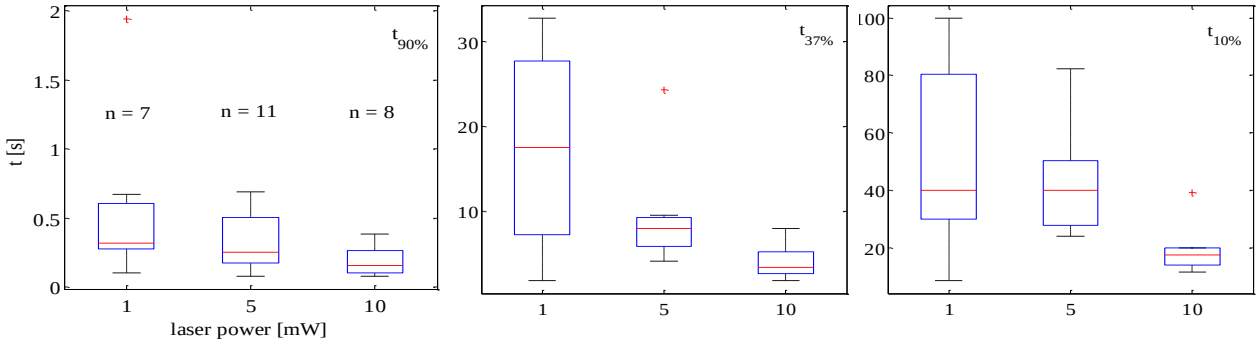

Fig. 4. Boxplots of photobleaching time coefficients obtained with 1, 5 and 10 mW laser power. Photobleaching time coefficients are inversely proportional to the laser power.

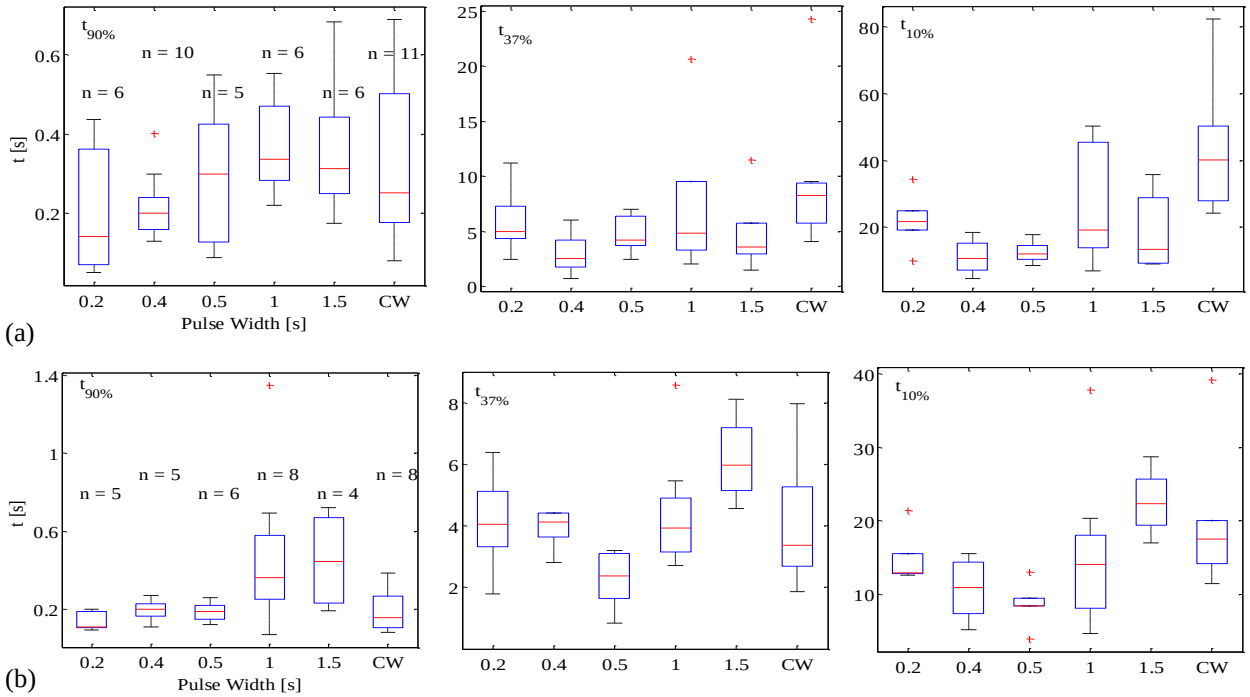

Fig. 5. Boxplots of time coefficients ($t_{90\%}$, $t_{37\%}$, $t_{10\%}$) of photobleaching in skin as a function of different pulse widths under (a) 5mW and (b) 10mW laser power. Statistical difference for 5 and 10 mW laser power is observed when 0.4 and 0.5 s pulsations were respectively used. This difference is not valid for $t_{90\%}$ as the values were small.

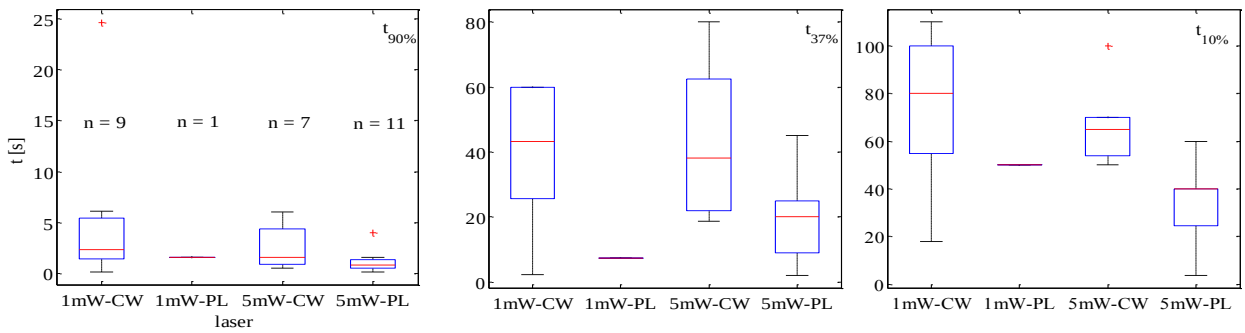

Fig. 6. Boxplots of photobleaching time coefficients measured in brain using 1 and 5 mW laser power both in continuous and pulsed modes. The photobleaching in pulsed mode shows a statistically significant difference in all three time coefficients compared to the similar continuous mode excitation ($p < 0.05$).

4 DISCUSSION AND CONCLUSION

Photobleaching of PpIX in skin and brain was studied using pulsed and continuous laser excitation light. Results from photobleaching of fluorescence at 635 nm is of interest when optimization of energy, a compromise of power and time, delivered to the tissue is discussed for the minimal photobleaching and maximal signal to noise efficiency. The pulsed energy in general, showed lower photobleaching time coefficients compared to continuous mode measurements.

The ratio number, quotient of PpIX fluorescence and tissue autofluorescence, is different for brain and skin tissue with a higher number in brain. This is explained by the generally low autofluorescence of brain but also by the different ALA administration methods used. Also, factors affecting the concentration of the PpIX in the cells and different optical properties of tissue may have an effect on the ratio number. Therefore, the photobleaching of PpIX in brain shows a slower time decay behavior compared to similar measurements in skin.

The energy delivered to the tissue has impact on the photobleaching, resulting in different time coefficients. The time coefficients for the pulsed laser illumination appeared shorter in comprison to continuous illumination. This effect has been discussed in photodynamic therapy to be a result of oxygen diffusion during the dark periods of the pulse as photobleaching is related to reaction of oxygen with PpIX [13, 14, 15]. However, dose and time coefficients were not found statistically significant as the non-illuminated intervals also affect the results. The change in tissue autofluorescence of ALA treated skin is obviously different from the non-ALA treated skin measurements. Same pattern is observed in glioblastoma. This might be due to the change in light absorption properties of the tissue when the PpIX molecules are decomposed.

In conclusion, a minimal energy of 2 mJ on brain offers an experimentally acceptable signal to noise ratio for stable measurements. The results show that photobleaching is affected by several factors such as excitation energy, PpIX concentration and oxygen availability in the tissue. In brain measurements, the 2.5 mJ pulsation showed significantly lower photobleaching time coefficients compared to similar continuous mode excitation ($p < 0.05$). Using the performed analysis, the dose which causes a photobleaching of only 10 % of the signal's initial fluorescence value can be estimated specifically for the continuous or pulsed mode fluorescence spectroscopy applications.

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