Noninvasive Murine Glioma Detection Improved Following Photobleaching of Skin PpIX Fluorescence

Summer L. Gibbs-Strauss¹, Scott C. Davis¹, Julia A. OHara¹,³, P. Jack Hoopes¹,², Tayyaba Hasan⁴, Brian W. Pogue¹,²,⁴
¹Thayer School of Engineering, ²Department of Surgery, ³Department of Diagnostic Radiology, Dartmouth College, Hanover New Hampshire 03755,
⁴Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA.

ABSTRACT

Aminolevulinic Acid (ALA) is a prodrug which can be administered to cells, animals or patients after which it is transformed via the Heme synthesis pathway into the fluorescent molecule Protoporphyrin IX (PpIX). PpIX has been shown to be useful as both a photosensitizer for photodynamic therapy (PDT) and as a fluorescence imaging contrast agent. The ALA-PpIX system not only provides contrast for fluorescence imaging but also gives information about the metabolic activity of the imaged tissue and thus could be useful for monitoring cancer therapy. In the current study skin photobleaching was examined to determine if PpIX fluorescence contrast in malignant brain tumors could be better visualized noninvasively. Red light photobleaching decreased skin PpIX fluorescence and increased the ability to noninvasively quantify PpIX fluorescence in murine gliomas, as in vivo measurements of mean PpIX fluorescence more closely matched ex vivo quantification following skin photobleaching. Three doses of blue light photobleaching (4 J/cm², 8 J/cm² and 12 J/cm²) were tested and determined to give similar levels of skin photobleaching as well as a similar window of decreased skin PpIX fluorescence for noninvasive fluorescence imaging following the photobleaching dose administration.

1. INTRODUCTION

Aminolevulinic Acid (ALA) is used as a prodrug, which after administration is converted into a fluorescently detectable product Protoporphyrin IX (PpIX) via the Heme synthesis pathway [1, 2]. The ALA-PpIX system is unique in the Photodynamic Therapy (PDT) and Photodynamic Diagnosis (PDD) world in that it is not only a photosensitizing system but is also a marker of cellular metabolism due to the enzymatic production of the fluorescent PpIX by the tissues of interest. The ALA-PpIX system has been studied extensively for PDT and PDD as well as other fluorescence imaging applications due to its unique nature of production as well as its rapid clearance from the body. ALA-PpIX based PDT has shown great success in dermatology applications such as treatment of actinic keratoses, Bowen’s disease, and basal cell carcinoma [3, 4]. ALA-PpIX based PDT and PDD of hollow organs with an epithelial lining such as the bladder, esophagus and cervix, have also shown promise [5]. PpIX production is also increased in malignant brain tumor tissue over normal brain tissue and has been used to guide neurosurgery of brain tumor resection [6-9]. Phase III clinical trials using PpIX fluorescence for neurosurgical guidance have been completed in Europe showing 6 month progression free survival to be higher in the ALA-PpIX fluorescence guided group of patient over patients receiving conventional white light guided surgery [7]. Although PpIX production is heterogeneous both between cell types and within cell lines, it is generally accepted that PpIX accumulation in tumors is higher than in the surrounding tissues making it a useful photosensitizer and fluorescence imaging contrast agent [10].

Although the ALA-PpIX system has shown great promise in applications such as dermatology, brain surgery, hollow organ tumor treatment and visualization it has also been met with some difficulty for the use in noninvasive whole body imaging. Reflectance based imaging techniques where the light source and the detection system are located on the same side of the subject have been largely unsuccessful, due to high PpIX production in the skin. Reflectance imaging geometries provide surface-weighted images which are highly affected by skin fluorescence, thus not allowing for visualization of anything more than a few millimeters in depth [11]. Reflectance based imaging is largely useless for imaging subcutaneous tumors beneath PpIX fluorescent mouse skin as well as any orthotopic tumor which would be at greater depth. Transillumination imaging schemes, where the light source is placed opposite the detection source with the subject between the two, provide images with greater depth penetration than can be obtained with reflectance based imaging. Use of transillumination imaging geometry allows for visualization of tissues at more than a few
In the current study PpIX imaging for visualization of intracranial brain tumors using transillumination imaging was investigated. High PpIX skin fluorescence of mice administered ALA limited contrast, therefore, skin photobleaching was investigated as a means to improve brain tumor signal detection. Both red light photobleaching and blue light photobleaching were examined for their ability to increase the detectable contrast in the tumor tissue as compared to the normal tissues. In the red light photobleaching experiment, measurements were taken during the photobleaching dose administration to determine when skin PpIX fluorescence decreased. In the blue light photobleaching experiment three photobleaching doses were compared and reaccumulation of PpIX fluorescence was tracked following the dose to determine if the skin photobleaching effect would last long enough for noninvasive tumor imaging to be completed. In the blue light photobleaching experiment measurements were not taken during the administration of the photobleaching dose.

2. MATERIALS AND METHODS

Murine Glioma Model – Two glioma tumor models were used in the current study; the 9L-GFP rat gliosarcoma cell line that had been transfected with green fluorescent protein (GFP) [12], supplied to us by A. Bogdanov and the U251 human glioma cell line. Both cell lines were used for intracranial tumor implantation in six week old male nude mice. The cells were implanted using a stereotactic frame and a Hamilton syringe loaded with 1 x 10^6 cells in 10 µl of PBS. All tumors were implanted 2 mm in front of the bregma, 2 mm to the left of the midline and 2 mm deep into the brain tissue. Sham surgery was performed on all control mice, where the same procedure was completed including injection of 10 µl of PBS into the brain tissue, but without tumor cells. Tumors were visible by T1 turbo spin echo (TSE) contrast enhanced (CE) MRI and T2 TSE MRI 10–14 days following implantation.

Transmission Fluorescence Spectroscopy – A single channel and multichannel spectroscopy system were used to obtain fluorescence measurements in the current study. The single channel spectroscopy system consisted of a 635 nm helium-neon laser, an Acton Research Spectrometer, a 650 nm LP filter, a computer for signal detection, and a light-tight box for the mouse. The laser light was collimated onto the chin of the mouse to excite PpIX, while a second collimator on top of the head of the mouse collected the transmitted signal. This signal was then passed through a 650 nm LP filter and into the spectrometer. The spectrum was detected using a commercially available software package, WinSpec (Acton Research, Acton, MA). Fluorescence emission data was obtained by centering the spectrometer at 705 nm, prior to any movement of the mouse transmittance data was also obtained by centering the spectrometer at 617 nm to obtain a spectrum of the laser peak.

The multichannel spectroscopy system consisted of two carts with 8 spectrometers on each cart, a 635 nm diode laser which was coupled into a rotary stage. The source fibers from the rotary stage were bifurcated into the detector fiber bundles which run from the individual spectrometers. Each spectrometer had a filter wheel containing a 650 nm LP filter for the detection of PpIX fluorescence. The system was controlled through LabView based software. Fluorescence emission data was obtained by centering the spectrometer at 705 nm and transmittance data by centering the spectrometer at 620 nm without movement of the mouse.

A two step post-processing was completed on both the fluorescence and transmittance raw data sets from either spectroscopy system prior to reporting results. During the data collect, exposure times were adjusted to obtain between 20,000 and 60,000 photon counts, which allowed for detection in the linear range of the spectrometer. To account for the required difference in data collection time, all data sets were normalized to exposure time so they could be directly compared in counts/second. The fluorescence data was spectrally fitted using PpIX phantom data so that the fluorescence signal could be deconvolved from any signal that leaked through the filters during measurements. The deconvolution of the fluorescence signal from extraneous filter bleed through signal via spectral fitting allowed the area under the fluorescence curve to be calculated and used as a single number to be compared between mice. The transmittance data was used to calculate the area under the laser peak. The integrated spectrally fitted fluorescence measurements were then divided by the integrated transmittance measurements giving the fluorescence/transmittance ratio.

Red Light vs. Blue Light Photobleaching – Two skin photobleaching studies were completed to explore PpIX brain tumor detection improvement with decreased skin PpIX fluorescence. A red-light time-course photobleaching study which included four control mice, nine 9L-GFP tumor-bearing mice and seven U251 tumor-bearing mice was...
completed using the single channel spectroscopy system. Endogenous PpIX fluorescence for each mouse was measured prior to administration of ALA. Following endogenous PpIX measurements 100 mg/kg ALA was administered i.p. Two hours after ALA administration, PpIX fluorescence was again measured. After the exogenous PpIX fluorescence measurement at two hours after ALA administration the 635 nm laser was left running so that skin photobleaching would occur. Additional PpIX fluorescence measurements were taken after 1, 2, 4, 8, 16, 24 and 32 minutes of photobleaching time. After the last photobleaching time point (32 minutes), the mice were sacrificed, their brains extracted and additional fluorescence spectroscopy measurements were taken on the bulk brain tissue *ex vivo.*

A blue light photobleaching study was completed on normal, healthy male nude mice using the multichannel spectroscopy system. In this study, three doses of photobleaching were compared (4 J/cm², 8 J/cm² and 12 J/cm²). A photobleaching apparatus was constructed which consisted of two high powered blue LED’s embedded in large heat sinks with a mouse platform in between the LED’s so that light could be delivered to both sides of the head simultaneously (figure 1). There were seven mice in the 4 J/cm² group, seven mice in the 8 J/cm² and eight mice in the 12 J/cm². Endogenous PpIX fluorescence measurements were obtained prior to the administration of ALA. Following endogenous PpIX measurement, 100 mg/kg ALA was administered i.p. Two hours after ALA administration the PpIX fluorescence of the mice was again measured. The mice were then removed from the spectroscopy system and placed in the photobleaching apparatus where the appropriate light dose was delivered. Following completion of the photobleaching, the mice were placed back in the spectroscopy system and measurements of PpIX fluorescence were obtained every two minutes for 32 minutes.

![Photobleaching apparatus including 2 LED's with large heat sinks with a mouse bed between them.](attachment:image1.png)

**Figure 1** - (a) Photobleaching apparatus including 2 LED’s with large heat sinks with a mouse bed between them. (b) Close-up of LED in aluminum heat sink (c) Photobleaching apparatus in operation.

3. RESULTS

*Red Light Photobleaching Time Course Study* – Tumor-bearing mice were compared to control mice in this study prior to ALA administration, 2 hours after ALA administration and during the photobleaching process to determine when the
highest contrast was visible between tumor bearing and non-tumor bearing groups. Sample normalized PpIX fluorescence/transmittance ratio spectra for a representative mouse in the control group, the 9L-GFP group and the U251 group can be seen in figure 2. Prior to ALA administration the mice in the non-tumor bearing control group and the mice in both tumor bearing groups had similar endogenous PpIX fluorescence. Two hours after ALA administration the 9L-GFP group had a slightly higher exogenous mean fluorescence than the U251 group and both tumor bearing groups had much higher exogenous PpIX mean fluorescence than the control group (figure 3(a)). The laser was left running after the initial exogenous PpIX fluorescence measurement and PpIX fluorescence decrease was measured after 1, 2, 4, 8, 16, 24 and 32 minutes of red light photobleaching. Figure 3(a) illustrates that the PpIX fluorescence significantly decreased during the photobleaching session; however contrast between the two tumor bearing groups became more significant during the photobleaching session. Figure 3(b) shows the percentage difference between the average of the control group and the average of each individual tumor bearing group. At 2 hours after ALA administration as well as after 1, 2 and 4 minutes of photobleaching the difference between the control group PpIX fluorescence and each tumor bearing group were very similar, with the 9L-GFP group showing slightly higher PpIX fluorescence than the U251 group. However, the measurement of PpIX fluorescence after 8 minutes of photobleaching illustrated that the U251 group actually had a higher mean PpIX fluorescence than the 9L-GFP group. This trend continued throughout the remainder of the experiment and was easily visualized when comparing the ex vivo measurements, where the U251 group shows over 700% difference from the control group while the 9L-GFP group only shows about 290% difference from the control group (Figure 3(b)). Thus, photobleaching the skin fluorescence increased the ability to detect the PpIX signal from the brain tumors without extraneous detection of skin PpIX fluorescence signal.

Figure 2 – PpIX spectrum at each measured time point for a representative mouse in the (a) 9L-GFP group (b) U251 group and (c) control group. The background measurement corresponds to the endogenous PpIX fluorescence, 2 hours is the initial exogenous PpIX fluorescence, and the minutes represent the PpIX measurement after that amount of photobleaching has been completed.
**Figure 3** - (a) Red light PpIX photobleaching time course. Time = -2 represents the endogenous PpIX fluorescence measurement prior to the administration of ALA. Time = 0 represents the initial exogenous PpIX fluorescence measurement 2 hours after that administration of ALA. All subsequent time points represent the amount of photobleaching time prior to the measurement. (b) Calculation of the percentage difference between the average of the control group and the average of each tumor bearing group at 2 hours after ALA administration (Time = 0) and all time points during the photobleaching session. Time = 35 minutes represents the *ex vivo* PpIX fluorescence measurement.

*Blue Light Photobleaching Dose Comparison Study* – In this study PpIX fluorescence of normal, healthy mice was compared prior to ALA administration, 2 hours after administration and then during the 32 minutes following a photobleaching dose of 4 J/cm², 8 J/cm² or 12 J/cm² to determine if skin PpIX photobleaching and PpIX reaccumulation were affected by light dose. Prior to ALA administration the mean PpIX fluorescence in each of the three photobleaching groups was similar (figure 5), although the variance in each of the groups was quite significant (figure 4). Two hours after ALA administration the skin PpIX fluorescence was measured to determine skin production levels. As can be seen in figure 5, each of the groups had similar mean PpIX fluorescence at 2 hours, although the variance between animals was again quite significant (figure 4). Following the exogenous PpIX fluorescence measurement the mice were administered 4 J/cm², 8 J/cm² or 12 J/cm² of blue light using the photobleaching apparatus (figure 1) and were immediately replaced into the multichannel spectroscopy system for post-photobleaching monitoring. Measurements were acquired every 2 minutes for 32 minutes after the photobleaching dose was delivered. Figure 5 illustrates that the skin PpIX fluorescence decrease was quite significant for all groups as compared to the initial exogenous skin PpIX fluorescence measurement two hours after ALA administration, prior to photobleaching. However, little significant difference was seen between the mean PpIX fluorescence of the 4 J/cm², 8 J/cm² or 12 J/cm² groups following the photobleaching dose, indicating that 4 J/cm² was sufficient to cause skin photobleaching and additional light dose was unnecessary. Figure 4 illustrates the heterogeneity in PpIX production in each of the photobleaching groups.
Figure 4 – Fluorescence/Transmittance Ratio After Blue Light Photobleaching. Individual mice shown in (a) 4 J/cm² photobleaching group, (b) 8 J/cm² photobleaching group and (c) 12 J/cm² photobleaching group. In all graphs Time = -2 represents the endogenous PpIX fluorescence measurement prior to ALA administration. Time = 0 represents the initial exogenous PpIX fluorescence measurement 2 hours after ALA administration. All subsequent time points represent the amount of time past after the photobleaching dose was administered.
4. DISCUSSION

In this study it was demonstrated that by decreasing skin PpIX fluorescence, there was increased ability to detect intracranial murine brain tumors. The method of skin PpIX fluorescence decrease used here was photobleaching the skin PpIX fluorescence with either red light or blue light. In the initial photobleaching experiment red light was used to administer the photobleaching dose by leaving the laser used for PpIX fluorescence measurements running in between fluorescence measurements, thus causing skin photobleaching. This allowed for monitoring of PpIX skin fluorescence decrease during the administration of the photobleaching dose, which proved to be quite significant over the 32 minutes of administration (figure 3(a)). Although the total PpIX fluorescence signal from the mice in the control group, the 9L-GFP group and U251 group was significantly decreased, the ability to quantify the fluorescence in the brain tumor tissue was improved by the red light skin photobleaching. As can be seen in figure 3(a), the PpIX fluorescence measurement 2 hours after administration of ALA illustrated that the mean PpIX fluorescence was highest in the 9L-GFP group, slightly lower in the U251 group and significantly lower in the control group. However, when the ex vivo situation was considered the mean PpIX fluorescence in the U251 group was about twice that of the mean PpIX fluorescence in the 9L-GFP group. Measurements taken during the photobleaching time course illustrated that the mean PpIX fluorescence of the 9L-GFP group was slightly higher than in the U251 group 2 hours after ALA administration as well as after 1, 2 and 4 minutes of photobleaching. However, the PpIX fluorescence measurements obtained following 8 minutes of photobleaching showed that the mean PpIX fluorescence of the U251 group was higher than that of the 9L-GFP group for the first time in the experiment. This relationship was seen throughout the remainder of the experiment as well as in the ex vivo case (figure 3(b)).

In the second portion of this study, blue light photobleaching was considered as an alternative to red light photobleaching. Although the red light photobleaching did decrease skin PpIX fluorescence and improve murine glioma detection, due to its deep penetration depth it may have also photobleached the brain tumor as well and thus blue light was considered as an alternative with a relatively shallow penetration depth in tissue [13]. In the blue light photobleaching experiment normal, healthy mice were considered so that skin photobleaching dose could be assessed for efficacy without the confounding brain tumor fluorescence. In this study, three photobleaching doses were considered including 4 J/cm², 8 J/cm² and 12 J/cm² to determine if there was a difference in skin photobleaching as well.
as the time to PpIX reaccumulation with the varied photobleaching dose. Ideally, a low dose of photobleaching such as 4 J/cm² would be sufficient to cause useful skin photobleaching since administration time would be less significant than at higher doses as well as a decrease in cutaneous phototoxicity following the photobleaching dose. There was high variability between animals in each of the photobleach groups (figure 4), however when the mean PpIX skin fluorescence was averaged in each group the endogenous PpIX fluorescence prior to ALA administration as well as the exogenous PpIX fluorescence 2 hours after ALA administration were very similar (figure 5). The three photobleaching doses caused similar amounts of skin PpIX photobleaching (figure 5). All three photobleaching doses also decreased skin PpIX fluorescence for the 32 minutes the mice were monitored following the photobleaching and did not show any significant PpIX reaccumulation in the skin. This 32 minute window of decreased skin PpIX fluorescence would allow for imaging to be completed on intracranial brain tumor tissue during the period of decreased skin PpIX fluorescence.

In summary, noninvasive visualization of murine brain tumor tissue metabolism can be improved through photobleaching of extraneous skin PpIX fluorescence signals. Although red light photobleaching provided better tumor tissue detection over detection without photobleaching, its depth of penetration in photobleaching is unclear. Blue light photobleaching looks promising for future noninvasive detection of murine glioma tumor tissue metabolism and could be used as an integral step in visualization of PpIX fluorescence in orthotopic murine tumors.

5. ACKNOWLEDGEMENTS

This work has been funded by NCI grants RO1CA109558 and P01CA84203, as well as the Norris Cotton Cancer Center Shared Resources.

6. REFERENCES

