Photodynamic therapy (PDT) is used to cause specific tissue destruction, using photochemical reactions that result from light excitation of photosensitizer, which has localized in the target tissue. The process of photosensitizer photobleaching was investigated to redefine the approach to dosimetry, when applied to treatment of Barrett’s esophagus.
An animal model of induced Barrett’s esophagus was developed on Sprague-Dawley rats. An Esophagojejunostomy technique was used to generate chronic reflux with pancreaticobiliary and gastric secretion in lower esophagus. Rats with esophagojejunostomy surgery were survived through 5 months and were used in our photosensitizer kinetic study. Columnar epithelium was easily observed, characterized as hyperplasia, ulceration, inflammation and dysplasia. Our study demonstrated a feasible technique to induce Barrett’s esophagus model in rat.

In order to interpret real-time dosimetry parameters like photosensitizer concentration and light fluence rate, a passively monitoring in vivo fluorescence dosimeter was developed to measure dosimetry parameters during the PDT treatment. Both phantom and animal studies had been carried out to verify system stability and sensitivity.

The photosensitizer, 5-aminolevulinic acid (ALA) induced Protoporphyrin IX (PpIX), pharmacokinetic was investigated by several methods. PpIX concentration in both induced Barrett’s esophagus and normal esophagus was investigated and compared. A PpIX accumulation peak was observed at early hour post ALA administration. PpIX distribution heterogeneity was observed in esophagus and variations were found within individual animals.

Photobleaching and its effects on ALA-PpIX PDT of normal rat esophagus were investigated in detail. Applying the real-time dosimeter, PpIX photobleaching kinetics and light fluence rate kinetics were studied. A PpIX photobleaching model was developed
to interpret the relation between photobleaching kinetic, rate and tissue singlet oxygen
dynamic. The result suggested that PpIX photobleaching kinetic should be considered to
improve our dosimetry model accuracy.

Finally, a dosimetry model was re-defined as a function of real-time
photosensitizer concentration, light fluence rate and effective irradiation time. PpIX
photobleaching kinetic was applied to determine the effective irradiation time.
Optimization of individual treatments could become routine to decrease variability in
treatment response and should be applied in ongoing clinical trials.
Acknowledgements

This thesis could not have been completed without guidance and assistance of several people. I would like to thank my supervisors Dr. Brian W. Pogue and Dr. P. Jack Hoopes for their support, encouragement and guidance throughout the Ph.D. program.

My greatest gratitude goes to Brian, who helped me grow as a scientist. As part of his team, I was exposed to a very stimulating environment. I certainly appreciate the amount of time and effort he devoted to help me meet the challenges I faced during the last five years. His great personality and his fantastic enthusiasm, inexhaustible energy and amazing insight into the field challenged and motivated me to strive for perfection and innovation.

I must express my gratitude to Jack, who never stops offering help with his knowledge in the biomedical area. Jack was a great resource for discussing all sorts of theoretical and experimental issues, and he always seems to have time to offer practical advice. I really enjoyed the time sitting with Jack beside a microscope, discussing experiment results and future plans. His advice kept me on the right track.

I would like to acknowledge Prof. Tayyaba Hasan and Prof. Keith Paulsen for their support and suggestion during my studies. Prof. Hasan gave me the opportunity to work in her lab in the summer of 2005 to perform part of this thesis work. Her kindness and effort helped me a lot with my research.

I appreciated the great help from Jeffrey A. Bergeron, who helped me with the
Barrett’s esophagus model. Without his skill in animal surgery, this model could not be developed. I would like to thank Bin Chen, Rendall R. Strawbridge, Susan A. Kane, Mark Savellano and Julia A. O’Hara for their warm hearted advice and support on animal experiments, Summer Gibbs, Xiaodong Zhou, Dax Kepshire, Scott Davis, Heng Xu, Qianqian Fang, Xiaomei Song and Christina Skourou for their valuable discussions, Shudong Jiang for her family-style kindness and warmth. I also appreciated Gregory Burke, Rene Dauphinais, Roger Dauphinais and Michael J Ibey for their suggestions about the construction of the dosimeter.

Special thanks to Hui Chang, for her constant support in my everyday life and her artistic talent to draw the wonderful figures in this thesis.

Finally, I would like to thank my parents for their support and encouragement through these years. Without their support, I would not have made it to where I am today.

This work was supported by the NIH grant PO1CA84203 and Wellman Center for Photomedicine, Massachusetts General Hospital Graduate Education Funds.
# Table of Contents

Abstract ....................................................................................................................................... ii  
Acknowledgements ..................................................................................................................... v  
Table of Contents ......................................................................................................................... vii  
List of Figures ............................................................................................................................... x  

## Chapter I  Introduction and Overview ........................................................................ 1  
1.1 Definition of Photodynamic Therapy ........................................................................... 1  
    1.1.1 Basic Mechanisms in Photodynamic Therapy .................................................... 3  
    1.1.2 Advantages and Limitations of PDT ................................................................. 7  
    1.1.3 PDT Treatment Planning .................................................................................. 8  
1.2 Dosimetry in Photodynamic Therapy ......................................................................... 9  
1.3 5-Aminolevulinic Acid - Protoporphyrin IX Photodynamic Therapy for Ablation of  
    Barrett’s Esophagus ................................................................................................. 11  
1.4 Hypotheses .................................................................................................................... 13  

## Chapter II  Rat Model for Barrett’s Esophagus ............................................................ 16  
2.1 Definition of Barrett’s Esophagus ............................................................................. 16  
    2.1.1 Characteristics of Barrett’s Esophagus ........................................................... 18  
    2.1.2 Factors Induce Barrett’s Esophagus ................................................................. 21  
2.2 In Vivo Rat model of Barrett’s Esophagus ............................................................... 22  
2.3 Results and Analysis ................................................................................................. 28  
    2.3.1 Rats Health Analysis Post Esophagojejunostomy Surgery ............................... 28  
    2.3.2 Pathology Study of Rats Esophagus Post Surgery ........................................... 30  
2.4 Conclusions ............................................................................................................... 35  

## Chapter III  Development of a Passive Dosimetry System for the Esophagus .......... 36  
3.1 Fluorescence Measurement of 5-Aminolevulinic Acid Induced Protoporphyrin IX  
    ................................................................................................................................... 36  
3.2 Description of a Passive Esophagus Dosimetry System .......................................... 38  
3.3 System Calibration in Liquid Phantom ..................................................................... 44  
    3.3.1 Light Dosimeter Calibration ............................................................................. 46  
    3.3.2 Correcting for Signal Cross-talk ..................................................................... 46  
    3.3.3 Photosensitizer Dosimeter Calibration ............................................................ 48  

## Chapter IV  5-Aminolevulinic Acid Induced Protoporphyrin IX Pharmacokinetics in  
    Normal/Barrett’s Rat Esophagus .............................................................................. 52  
4.1 Introduction of 5-Aminolevulinic Acid Induced Protoporphyrin IX ............................ 52  
4.2 ALA-PpIX Pharmacokinetics in Rat Normal Esophagus ........................................ 55
4.2.1 Materials and Methods...................................................................................... 55
4.2.2 Results and Analysis ......................................................................................... 57
4.3 ALA administration Methods in Normal/Barrett’s Rat Esophagus: Topical vs. Systematical Administration.......................................................................................... 60
4.3.1 Materials and Methods...................................................................................... 60
4.3.2 Results and Analysis ......................................................................................... 62
4.4 Discussion ............................................................................................................... 67

Chapter V Protoporphyrin IX Photobleaching and Its Effects on Photodynamic Therapy in Normal Rat Esophagus............................................................................... 75
5.1 Mechanism of Protoporphyrin IX Photobleaching ................................................. 77
5.2 Materials and Methods ............................................................................................ 80
5.3 Results and Analysis .............................................................................................. 84
5.3.1 ALA-PpIX-PDT induced damage ..................................................................... 84
5.3.2 Fluence rate measurements ............................................................................... 88
5.3.3 PpIX photobleaching ........................................................................................ 89
5.3.4 Effects of PpIX photobleaching in continuous PDT treatment.......................... 91
5.3.5 Effects of PpIX photobleaching in fractionated PDT treatment ......................... 92
5.3.6 PpIX Photobleaching with PDT Response ....................................................... 94
5.4 Discussion of PpIX photobleaching and O$_2$ Consumption in PDT Treatment..... 96

Chapter VI Real-time Photodynamic Therapy Dosimetry ...................................... 101
6.1 Photodynamic Therapy Dosimetry ........................................................................ 101
6.2 Materials and Methods .......................................................................................... 104
6.3 Results and Analysis .............................................................................................. 107
6.3.1 ALA-PpIX PDT response assessment ............................................................ 107
6.3.2 PpIX uptake in PDT dosimetry ......................................................................... 110
6.3.3 Using Real-time PpIX kinetics in PDT dosimetry ........................................... 112
6.3.4 Analysis of the effective treatment time in PDT dosimetry .............................. 115
6.4 Discussion of Effective PDT Dosimetry ............................................................... 118

Chapter VII Conclusion and Future Works .............................................................. 124
7.1 Thesis summary ..................................................................................................... 124
7.2 Future work ........................................................................................................... 127
7.2.1 Topical administration of ALA ....................................................................... 127
7.2.2 PpIX photobleaching and singlet oxygen in situ ............................................. 128
7.2.3 Dosimetry controlled PDT treatment .............................................................. 129

APPENDIX A in vivo Dosimeter System .................................................................... 131
A. Data Acquisition Hardware. ................................................................................... 131
B. Data Acquisition Software .................................................................................... 132
BIBLIOGRAPHY

................................................................................................................................. 135

ix
List of Figures

Figure 2.1 illustrates the anatomical changes following the esophagojejunostomy surgery. (a) is the anatomy of normal rat upper GI track, esophagus connected to stomach. (b) esophagus connected to jejunum approximately 3 cm posterior to the duodenum……………………………………………………………………………………………  24

Figure 2.2 Surgical creation of a rat model of Barrett’s esophagus. (a) The gastroesophageal junction (white arrow) was identified and isolated for esophagus transection. (b) The vagus trunk (white arrow) was preserved during transaction. (c) A 5-mm jejunostomy was created approximately 3 cm post duodenum. (d) While carefully maintaining the orientation and patency of the lumen, an end-to-side esophagoenterostomy was performed………………………………………………………………………………..25

Figure 2.3 Esophagus and jejunum anastomosis of rat (57 days post surgery). (a) Anastomosis (white arrow) lateral with stomach, this figure illustrate the similar organ positions in rat body. (b) Anastomosis (white arrow) is located approximately 3 cm posterior to the duodenum (dark colored on the left). This anastomosis permitted both duodenal and gastric reflux into the esophagus……………………………………………………………..27

Figure 2.4 Rat weight changes post esophagojejunostomy surgery. Blue solid lines show the weight change of 11 survival rats, red dashed lines show the weight change of 3 dead rats with failed surgery (death date 11 14 and 15). There was significant weight drop at the first 2-5 days due to the fasting procedure 48 hr pre surgery and 24 hr post surgery. With the food and recovering from surgery, rats began to gain weight after 2 weeks post surgery. Rats regulated about 2 weeks to fully recover from surgery and begin to gain weight……………………………………………………………………………………………..29

Figure 2.5 Esophagus epithelium changes 57 days post esophagojejunostomy surgery. (a) Macro view of esophagus anastomosed to jejunum shows ulcerative and hyperplastic esophagitis at the lower esophagus. (b) 40× microscopy sections shows columnar epithelium was formed at the lower esophagus…………………………………………..31

Figure 2.6 4× microscopy sections of esophagus with H&E stain. (a) Normal rat esophagus. (b) esophagus extracted from rat at 68 days post surgery. Compared to the normal esophagus, the hyperplastic reaction was confined to the mucosa, causing it to become 3-4 times larger than normal. Ulceration was observed (the upper portion in figure d)……………………………………………………………………………………………..32

Figure 2.7 40× microscopy sections of esophagus with H&E stain. (a) Normal rat esophagus. (b) 57 days post surgery. (c) 68 days post surgery. (d) 152 days post surgery.
Compared to normal esophagus with neat even epithelium, esophagi with anastomosis to jejunum show columnar disorder epithelium layers. Dysplasia was found in all three cases, characterized by hyperchromatic, enlarged nuclei and depletion of cytoplasmic mucin. Inflammatory cells were also observed...

Figure 2.8 40× microscopy sections of esophagus with H&E stain. (a) published illustration of human Barrett’s esophagus section (www.barrettsinfo.com). (b)-(f) rat esophagus sections extracted 30 weeks post surgery. Columnar disorder epithelium layers and dysplasia were found in most cases (b-e), characterized by hyperchromatic, enlarged nuclei and depletion of cytoplasmic mucin. (f) Barrett’s esophagus was not seen in this esophagus section. The epithelium was not altered...

Figure 3.1 The emission spectrum (excited at 635 nm) of Protoporphyrin IX (PpIX) is shown in saline, as acquired by a cuvette-based spectrophotometer (SPEX FluoroMax-3). The solid lines are the PpIX spectrum before PDT irradiation. Dashed lines are fluorescence (may include photoproduct from PpIX photobleaching) spectrum after 10 J/cm² PDT irradiation. PpIX photobleaching product has a fluorescence peak around 670 nm while PpIX itself has a fluorescence peak around 700 nm...

Figure 3.2 Passive dosimetry structure, including a brief illustration of the fiber excitation at 635 nm and the way to split the signal of the detection fibers into 690 long pass and 620-650 bandpass, allowing simultaneous detection and rationing...

Figure 3.3 The overview and key components of the dosimetry system are shown. (a): The dosimetry system is comprised of the 6 to 1 optical switch (upper-left), signal detector (lower-left), electronic control board (upper-middle) and DAQ card (lower-right). (b): The 6 to 1 optical switcher will receive 6 input optical signals and transfer these signals to the detection part sequentially. (c): The fluorescence spectrum of PpIX excited by 635nm. The PpIX has a fluorescence peak around 700nm, and this fluorescence peak will be used in our dosimetry system to measure the PpIX concentration. (d): The detection part includes two PMTs and two sets of filters, the optical signal will be divided into two channels and give us the information about the PDT irradiation signal and PpIX fluorescence signal.

Figure 3.4 (a) The MR image for the pig esophagus and the balloon probe is shown. In (b) the esophagus with the dosimetry balloon probe is shown, with (c) the dosimetry balloon probe for the pig esophagus, and (d) the dosimetry probe used for the rat esophagus.

Figure 3.5 User interface for in vivo dosimeter system. Data filename and experiment comments were setup at the left screen and measure curve/data was displayed at the right
Figure 3.6 Correlation of reference channel signals to the laser output is shown. Measurements were taken in liquid phantoms with the similar optical properties as $\mu_s'=1.1\text{mm}^{-1}$ and $\mu_a=0.04\text{mm}^{-1}$. No Protoporphyrin IX was added in phantoms. Laser output changes from 0 mW/cm to 165 mW/cm. The reference channel PMT output signals (voltage) has linear correlation with laser outputs.

Figure 3.7 Crosstalk from reference channel to fluorescence channel. Each solid line refers to an set of phantoms (same reduced scattering coefficient $\mu_s'=1.1\text{ mm}^{-1}$ and different absorption coefficient $\mu_a = 0.0125, 0.025, 0.04$ and $0.05\text{mm}^{-1}$). No PpIX was inside the phantoms, and the laser output changed from 32 mW/cm to 128 mW/cm. Within the expected ranges of absorption coefficient, the crosstalk remain consistent to within 6% standard deviation.

Figure 3.8 Two channel PMTs Readings of liquid phantom with different Protoporphyrin IX concentrations. Measurements were taken in liquid phantoms with the similar optical properties as $\mu_s'=1.1\text{mm}^{-1}$ and $\mu_a=0.04\text{mm}^{-1}$. Protoporphyrin IX concentrations change from 0 ug/ml to 3 ug/ml. Blue dots are refer to fluorescence channel PMT readings, while red crosses are refer to reference channel PMT readings. The reference channel PMT reading did not show positive correlation with Protoporphyrin IX concentrations, which indicated a minor crosstalk effect of the fluorescence channel into the reference channel. The reference channel signal could be considered independent.

Figure 3.9 Correlation of fluorescence channel signals (after crosstalk adjustment) to PpIX concentrations. This figure illustrated a positive linear correlation between dosimeter system readings and Protoporphyrin IX concentrations in liquid phantom. The detection precision was 0.1 ug/ml Protoporphyrin IX, and the linear range is up to 4 ug/ml.

Figure 4.1 The heme biosynthetic pathway is illustrated schematically (A, Ferrochelatase with Fe2+; B, PBG-deaminase).

Figure 4.2 PpIX fluorescence signal in normal rat esophagus tissue as measured in situ by two methods: the in vivo fluorescence dosimeter (a) and the ex vivo fluorescence scanner (b). Both methods yields similar PpIX uptake kinetic. PpIX in the esophagus has an uptake peak at early hours post ALA administration and drops back to background level at 24 hour. (*p-value=0.0004, # p-value=0.0002) (Animal used in control: n=5; 2hr: n=6; 4hr: n=6; 24hr: n=6).

Figure 4.3 PpIX signals measured from plasma measured in the spectrometer. The PpIX
fluorescence in the plasma increased after the ALA administration and stayed at a high level for to the duration of the 24 hour period studied here. (Animal used in control: n=5; 2hr: n=6; 4hr: n=6; 24hr: n=6).……………………………………………………………………………………………………59

Figure 4.4 The surgical procedure demonstrated in this diagram was used to occlude the esophagus for administrating ALA in a topical manner. Topical administration of ALA in is illustrated. in (a) & (b) for the normal esophagus, and (c) & (d) showing the same procedure for the Barrett’s esophagus rats. The red line in (a) and (c) illustrates the suture position to isolate the esophagus. And blue arrows in all 4 figures illustrate the location where the ALA injection was done……………………………………………………………..61

Figure 4.5 Figure 4.5 Fluorescence images acquired by fluorescence scanner (Typhoon 9410, GE) 2hr post ALA administration with a dose of 100 mg/kg. Shown are (a) the control group (n=6); (b) normal rat esophagus with i.p. ALA administration (n=7); (c) normal rat esophagus with topical ALA administration (n=7); (d) rat Barrett’s esophagus with i.p. ALA administration (n=3); (e) rat Barrett’s esophagus with topical ALA administration (n=4).…………………………………………………………………………63

Figure 4.6 The average PpIX fluorescence in esophagus at 2hrs post 100 mg/kg ALA administration. There was an increase of PpIX fluorescence with the ALA administration. Different administration methods did not show significant differences in either normal esophagus or induced Barrett’s esophagus. (p-value are listed in the text above, and animal used in group control: n=6; i.p. in normal: n=7; topical in normal: n=7; i.p. in BE: n=3; topical in BE n=4).…………………………………………………………64

Figure 4.7 Fluorescence images as acquired by the fluorescence scanner (Typhoon 9410, GE) 2hr post ALA administration with a dose of 100 mg/kg. In each image, the upper row is kidney sections and lower row is liver sections. In (a) is the control group (n=6); (b) the group with normal esophagus and i.p. ALA administration (n=7); (c) the group with normal esophagus and topical ALA administration (n=7); (d) the group with induced Barrett’s esophagus and i.p. ALA administration (n=3); and (e) the group with induced Barrett’s esophagus and topical ALA administration (n=4).……………………………………………………………..65

Figure 4.8 PpIX fluorescence in the kidney and liver 2hrs post 100 mg/kg ALA administration. There was an increase of PpIX fluorescence in the liver with i.p. ALA administration as well as in the Barrett’s esophagus group. Different administration methods did show significant difference in liver for normal esophagus groups (p-value=0.013). But no significant difference was observed in the liver within the Barrett’s esophagus groups (p-value = 0.50 and animal used in group control: n=6; i.p. in normal: n=7; topical in normal: n=7; i.p. in BE: n=3; topical in BE n=4).………………………………………66
Figure 4.9 (a) PpIX fluorescence macro images of Barrett’s esophagus after 100 mg/kg ALA administration, where the letters b-f mark the approximate positions where microscopic images were taken. In (b)-(f) the microscopic images with H&E stain, related to the marked area of b-f in (a). 72

Figure 4.10 PpIX fluorescence intensity has positive dependence on the esophagus columnar epithelium height. Total 7 animals with Barrett’s esophagus model was under investigation here. 73

Figure 5.1 Macro views of normal esophagus 48 hours post ALA-PpIX PDT. In (a) the rat in control group A is shown, without any ALA administration and light irradiation. In (b) the rat in control group C is shown, without any ALA administration but with 20 J/cm light irradiation at 50 mW/cm. In (c) the rat in treatment group F is shown with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at a fluence rate of 50 mW/cm. In (d) the extracted individual esophagus from (a), (b) and (c) are shown from top to bottom. In the control groups no damage was observed, but in the treatment groups, the esophagus wall was inflamed. 85

Figure 5.2 The microscopic view of H&E stained cross-sections of normal esophagus, 48 hour post ALA-PpIX PDT. In (a) a rat in control group A is shown, without any ALA administration and light irradiation. In (b) and (c) cross sections of esophagus are shown from a rat in treatment group F with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 50 mW/cm. Since the PDT treatment laser fiber had a 1 cm diffusing tip end, about 1 cm of the lower esophagus was the irradiated area, shown in (c). While the upper esophagus received no light irradiation, so was considered as un-irradiated area, shown in (b). The treatment response/damage was significantly different between the irradiated area and un-irradiated areas in all individual animals. Compared to the control group, the esophagus in the un-irradiated area received almost no damage while a large area of edema appeared in the irradiated zone. In (d) showed a high magnification view (40X) of a rat esophagus in the group with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 25 mW/cm. Ablation of epithelium was observed in this case. 86

Figure 5.3 Cross sectional edema area of the esophagus is shown. The blue bars refer to mucosa/submucosa area in lower esophagus, where light was applied and subsequent enlargement was observed. The dark red bars refer to mucosa/submucosa areas in upper esophagus, where edema was rarely observed and the mucosa/submucosa area was at same value throughout all groups (including control group). For the lower fluence rate group, edema area was larger compared to higher fluence rate group. The fractionated treatment group yielded increased edema as compared to the continuous treatment group. (Animals used in group A: n=6; B: n=6; C: n=6; D: n=7; E: n=7; F: n=7; G: n=7; H: n=7;
Figure 5.4 In vivo PDT treatment light fluence rate changed during treatment with a maximum variation of 17%. The mean value of the fluence rate was similar to the initial in vivo light fluence rate set up (50 mW/cm and 25 mW/cm, respectively). (Animals used in group C: n=6; D: n=7; E: n=7; F: n=7; G: n=7; H: n=7; I: n=7).

Figure 5.5 In vivo PpIX kinetics are shown during light delivery, where the dots are experiment data points and solid line is the monoexponential decay fitting to the early phase of PpIX photobleaching.

Figure 5.6 PpIX photobleaching rates fitted to the first phase of PpIX decay. There is no significant fluence rate dependence for the first phase PpIX photobleaching, and the overall mean value is 0.029 ± 0.013 cm/J. (Animals used in group D: n=7; E: n=7; F: n=7; G: n=7).

Figure 5.7 PpIX kinetic in fractionated PDT. Repeating of rapid decay phase in each PDT irradiation fraction was observed. And photobleaching rates in each irradiation fraction were at similar value level.

Figure 5.8 Photobleaching rates in individual animals in the fractionated PDT group. The average photobleaching rate is higher for fractionated irradiation than for continuous irradiation, but there were still some irradiation fractions that had low photobleaching rates, resulted in large standard deviation bars. The fractionated PDT was done with 2 fluence rates 50 mW/cm (a) and 25 mW/cm (b).

Figure 5.9 Plot of PpIX photobleaching rate and PDT response (edema area). The blue stars refer to animals in continuous PDT groups, and green circles refer to animals in fractionated PDT groups. The red solid line is the regression line to the data, the correlation between PpIX photobleaching rate and PDT response is 0.19 (p-value=0.319, and animal number used in this experiment is 30).

Figure 6.1 The 10× microscopic view of the H&E stained cross-sections of normal esophagus, 48 hour post ALA-PpIX PDT. This set of images illustrates a wide range of PDT response. Compared to the control group rat (a), some rats with ALA administration and light irradiation show different PDT responses including no damage (b), edema in the mucosa/submucosa area (c) and epithelial cell ablation (white arrow) in (d).

Figure 6.2 The rat esophagus cross sectional edema assessment is shown. The blue bars
refer to mucosa/submucosa area in lower esophagus, where light was applied and subsequent enlargement was observed. The dark red bars refer to mucosa/submucosa areas in upper esophagus, where edema was rarely observed and the mucosa/submucosa area was at same value throughout all groups (including control group). For the lower fluence rate group, edema area was larger compared to higher fluence rate group. The fractionated treatment group yielded larger edema area compared to the continuous treatment group.

Figure 6.3 The results of PpIX fluorescence signal from rats with different ALA i.p. administration doses (0 mg/kg, 50 mg/kg and 100 mg/kg). In the rat esophagus with exogenous ALA, the average PpIX uptakes are nearly 2 fold as large as the control groups, even with standard deviation levels of 17% to 21%.(Animal used in normal group: n=12; 50 mg/kg ALA injection group: n=28; and 100 mg/kg ALA injection group: n=20).

Figure 6.4 Illustration of the lack of correlation between PDT response (edema area) and the simple model for PDT dose is shown, which was calculated with the initial PpIX concentration in each animal. Each data point is an individual animal. The correlation coefficient is 0.06 Illustration of the lack of correlation between PDT response (edema area) and the simple model for PDT dose is shown, which was calculated with the initial PpIX concentration in each animal. Each data point is an individual animal. The correlation coefficient is 0.06 (p-value=0.757 and animal number used in this model is 30). Further modification is needed to optimize PDT dose calculation.

Figure 6.5 The kinetics of PpIX and fluence rate during PDT treatment in one rat. The animal received 50 mg/kg ALA i.p. administration and light irradiation at 25 mW/cm for 800 seconds. The PpIX signal decreased very quickly from 3.4 ug/ml to 2.2 ug/ml during the treatment, while the light fluence rate changed very little overall, within 23.6 mW/cm to 24.4 mW/cm. (Animals used in group A: n=6; B: n=6; C: n=6; D: n=7; E: n=7; F: n=7; G: n=7; H: n=7; I: n=7).

Figure 6.6 An illustration of the poor correlation between PDT response (edema area) and PDT dose, which is calculated by integrating the PpIX signal with the fluence rate in individual animals. Each data point is an individual animal. The correlation coefficient is 0.09 (p-value=0.881 and animal number used in this model is 30), which indicates a poor correlation. Further modification is needed to yield a PDT dose calculation that is predictive of response.

Figure 6.7 Two situations were observed in the PDT treatment groups, with 12 out of 42 rats not showing any PDT damage even when having high PpIX signals and light irradiation. In this situation, the PpIX kinetics (a) did not have a rapid decay phase after
irradiation and histology analysis did not find any PDT damage (b). PDT response (edema) was observed in another 30 rats in the treatment groups (d). In these 30 rats, a rapid decay phase of PpIX kinetics were observed (c), with the red line in (c) defining an effective transition point ending the rapid PpIX decay phase. This rapid decay phase could be considered as an effective PDT treatment period, because this phase is also considered to be the $^1O_2$ dependent photobleaching time, as discussed in Chapter V………………………………………………………………………………………...117

**Figure 6.8** The data is plotted to illustrate the correlation between PDT response (edema area) and the “effective” PDT dose, which is calculated by integrating the PpIX signal with the fluence rate in individual animals, but only for the duration of the rapid decay phase. The correlation coefficient is 0.45 (p-value=0.029 < 0.05 and animal number used in this model is 30), which indicates positive linear relationship between this modified PDT dose and response…………………………………………………………………………………………………….118

**Figure 6.9** Photobleaching rate histograms in two PDT response situations: PDT damage (edema) observed in 30 rats (a) and PDT damage NOT observed in 12 rats (b). In situation (a), the photobleaching rate was 0.029 ± 0.013 cm/J with a median of 0.027 cm/J. In situation (b), the photobleaching rate was 0.0042 ± 0.0059 cm/J with a median of 0.0018 cm/J………………………………………………………………………………………….120

**Figure 8.1** DAQCard™-6024E pin out schematic used………………………………………………………………………………………………………………………….132

**Figure 8.2** Figure 8.2 A flow chart of dosimeter measurement is shown……………….133
Chapter I  Introduction and Overview

Overall, the aim of our research project was to develop and refine a dosimetry model to optimize photodynamic therapy treatment of Barrett’s esophagus. In this study, 5-Aminolevulinic acid (ALA) induced Protoporphyrin IX (PpIX) was used as a model. The hypotheses of this thesis came from previous studies of photosensitizer distribution using an active fluorescence dosimetry system[1], this work is a comprehensive program involving a rat Barrett’s esophagus model development, dosimetry parameter measurements in tissue-phantoms and rats, along with appropriate modeling of the effects. Finally, a dosimetry model for ALA-PpIX PDT was developed and can be used in future research studies and clinical trial of PDT for Barrett’s esophagus.

To better understand the mechanism of our research work, there is an overview of photodynamic therapy, dosimetry in photodynamic therapy and current works in ALA induced PpIX PDT. The hypotheses of this thesis work were also listed in this chapter.

1.1 Definition of Photodynamic Therapy

Photodynamic therapy (PDT) involves the use of photosensitizers (PS), which are activated by certain wavelengths of light, to affect biochemical and hence biological and therapeutic changes in cells and tissues. According to the rigorous definition of the
photodynamic effect, the photochemical pathways involve oxygen as an essential component, a so-called Type II process [2]:

\[
S_0 + h\nu_e \rightarrow S_1 \\
S_1 \rightarrow T_1 \\
T_1 + ^3O_2 \rightarrow S_0 + ^1O_2 \\
^1O_2 + biom \rightarrow products
\]

where \( S_0 \) is the photosensitizer ground state, \( S_1 \) is the photosensitizer excited singlet state, \( T_1 \) is the photosensitizer excited triplet state, \( h\nu_e \) is the excitation photon, \( biom \) is any target bio-molecule, \(^3O_2\) is the ground state oxygen, and \(^1O_2\) is the singlet oxygen.

Most clinical PDT photosensitizers in use today are also thought to work in this way. However, preclinical studies of oxygen-independent methods of photoactivation are also in progress and some level of oxygen-independent damage can occur with certain photosensitizers.

Although many photosensitizers can be activated by light, the term PDT will be restricted to those in which the photochemical activation is the primary mechanism of action, and in which any biological effects occurring in the absence of the therapeutic light are incidental. PDT treatments typically use visible (400 to 700 nm) or near-infrared (700 to ~850 nm) light.

From the definition of PDT, it is evident that three major factors are involved in PDT treatment: photosensitizer, photons and oxygen in the local tissue. Investigation of the photosensitizer, photon and oxygen kinetics during PDT treatment will help improve the understanding of the mechanisms of PDT, and potentially improve PDT treatment.

2
plans to yield more PDT efficacy. This overall philosophy is predominant in this thesis.

1.1.1 Basic Mechanisms in Photodynamic Therapy

Photodynamic therapy (PDT) is based on the concept that certain photosensitizer drugs can be localized preferentially in neoplastic tissue, and subsequently can be activated with the appropriate wavelength of light to generate reactive molecular species that are toxic to the tissue. In this process, PDT is then a dual targeting therapy where the potential advantage is the inherent dual selectivity of drug and light localization. First, selectivity is achieved by an increased concentration of the photosensitizer in the target tissue, and secondly, the irradiation can be limited to a specified volume.

There are three main steps leading from light absorption by the photosensitizer to the therapeutic outcome, involving photophysical, photochemical and photobiological processes. If a single photon of certain wavelength is absorbed by the molecules in their ground state, the molecules are raised to excited singlet states. The excited state has a short lifetime, typically $\sim 10^{-9}$ s, and can decay in three ways: (1) non-radiatively, resulting essentially in heat; (2) by emission of a longer wavelength photon as fluorescence; or (3) by inter-system crossing to a triplet state, through electron spin rearrangement. The triplet state is relatively long-lived, typically $\sim 10^{-6}$ s. It can decay by phosphorescence, with light emission as in fluorescence, but this is a rare event. However, ground-state oxygen is also a triplet state, $^{3}\text{O}_2$, so that the probability is very high for
transfer of energy from the photosensitizer triplet state to oxygen. This produces excited singlet oxygen, $^1\text{O}_2$. The photosensitizer returns to the ground state, where it may undergo further activation cycles. Thus, the photosensitizer acts as a catalyst in the photo-production of $^1\text{O}_2$ from $^3\text{O}_2$. Singlet oxygen is highly reactive with and damaging to the neighboring biomolecules.

Due to its reactivity, $^1\text{O}_2$ has a short life time, typically significantly less than 1 $\mu$s in the tissue[3]. Since there is little time for $^1\text{O}_2$ diffusion, the biochemical damage occurs very close (10-20 nm) to the site of photosensitizer localization[4], where $^1\text{O}_2$ is produced. With many current photosensitizers, membranes are believed to be the primary binding site[5-8]. Depending on the kind of photosensitizer, its delivery mechanism, and the time for uptake and binding prior to light irradiation, the primary binding sites are thought to be the mitochondrial or other intracellular membranes[8]. The primary biochemical damage from $^1\text{O}_2$ is lipid peroxidation[9]. Subsequent results from the breakage of membranes also induce cellular toxicity through a heterogeneous and complex cascade of events.

If the concentration of $^1\text{O}_2$ is sufficient, then cell death occurs[10]. Depending on the conditions, necrotic and/or apoptotic cell death results[11-13], both in vitro and in vivo. The average cell survival rates strongly depend on the cell type, photosensitizer, and also vary with the incubation time and conditions, which affect the localization and binding of the sensitizer. Different cell types can be either very sensitive or quite resistant to PDT. No systematic differences in PDT sensitivity have been observed between tumor
and normal cells. Sensitivity to one photosensitizer does not imply sensitivity to other photosensitizers, so that the pattern of cell responses with different photosensitizers and incubation conditions is complex, and not completely understood in terms of systematic structure-function relations[2].

In the tissue response to PDT, two main types of damage mechanisms have been identified. These are: (1) direct target cell damage, for example, tumor cells[14], and (2) effects on tissue micro-vasculature[15]. The type of the damage mechanism which dominates the PDT effect depends on the tissue, photosensitizer and drug-light time interval. In the case of solid tumors, damage to blood vessels by killing of capillary endothelial cells is important with some photosensitizers. A complex pattern of response is often seen, including transient closing and opening of vessels. However, if the PDT dose is adequate, then vascular shutdown can occur, which causes ischemic tissue death to its immediate area. Part of the vascular response may itself be due to secondary effects from tumor cell death[15, 16].

Both local and systemic immunological effects are also known to occur with PDT[17]. For example, under some conditions the tissue response is more pronounced than can be accounted for by direct cell killing. It has also been shown that photosensitizer localization inside the solid tumor can be significant at site of macrophage involvement, which are known to have a role in the local tumor immunologic response to damage[18].

Many photosensitizers can be photobleached by light irradiation[19], i.e., the
concentration of photoactive drug falls as the drug is destroyed or altered in the photochemical reactions. There are two causes of photobleaching. One is due to the activation of the photosensitizer ground state itself, and the other is that the \(^1\text{O}_2\) causes the molecular damage to the sensitizer molecule that created it. For the latter, the more photosensitizer and light that are present, the more \(^1\text{O}_2\) is generated and the faster the photobleaching occurs. This will create a negative feedback by reducing the \(^1\text{O}_2\) production rate. Furthermore, for some photosensitizers, photobleaching generates photoproducts, which may absorb at a different wavelength, may be photoactive, or may be photobleached[20].

It is also known that the combination of high molar extinction coefficient and high concentration of photosensitizer can create significant absorption of light by the photosensitizer itself, such that it limits the light penetration in tissue[21]. In some extreme cases, as the photosensitizer concentration increases, the effective treatment depth can actually decrease. However, when the photosensitizer photobleaches, then the penetration increases again during treatment as photosensitizer nearest the surface is destroyed through bleaching loss.

It is important to re-emphasize that the mechanism of action in PDT can be highly dependent upon the photosensitizer, on the target tissue and on other treatment conditions. Thus, conclusions from preclinical and clinical trails may not translate directly from one photosensitizer or treatment protocol to another.
1.1.2 Advantages and Limitations of PDT

The general advantages of PDT for solid tumor therapy are[2]:

(1) It has double selectivity (photosensitizer and light), with either alone having little effect.

(2) It can be given before, after, or adjuvant to other standard therapies, including surgery, radiation and chemotherapy, with few known contraindications.

(3) There is a healing response of most normal tissues that are within the treatment field. This is thought to be due to the fact that PDT does not destroy the underlying collagen matrix structure of the tissue.

(4) It may be repeated as required for tumor control, without induction of either tumor resistance or normal tissue hypersensitivity.

(5) It has minimum side effects. The only significant concern has been generalized skin photosensitivity which requires the patient to take moderate precautions against bright light. With Photofrin, this sensitivity can persist for several months, but the effect is greatly reduced when second generation drugs are used, both in severity and in duration.

(6) By selection of the photosensitizer, delivery vehicle and drug-light time interval, different tissue components can be targeted and different mechanisms of action invoked for therapeutic gain.

The current limitations are[2]:
(1) PDT cannot easily be used for large tumor masses. The penetration of even longer-wavelength light in tissue limits the effective depth of treatment to typically 5 to 10 mm. Although multiple interstitial optical fibers can be used, as in branchytherapy, to increase this to much larger volumes, the very rapid tissue response from PDT can lead to complications, and only palliative use of this method of treatment of bulk tumors have been approved.

(2) The optimum photosensitizer and light doses and drug-light time interval may vary significantly from patient to patient, and even lesion-to-lesion in the same patient. Thus, standardized protocols likely do not achieve the maximum response rates possible, and patient-specific dosimetry is likely an area of need.

(3) Extensive pre-clinical and clinical trials are needed for each new photosensitizer and/or indication, and dosimetry protocols need to be specified for each drug and application, including specific dosimetry tools for the application.

1.1.3 PDT Treatment Planning

A large amount of development has taken place to produce photosensitizers that have optimal localization properties and have high absorption in the red or near-infrared region of the spectrum where there is optimal light penetration in tissue[22, 23]. However, beyond the scientific development of the photosensitizer, the process of establishing and planning new treatment protocols is difficult and has not taken advantage of new
dosimetry techniques. This lack of planning is attributable in part to the higher complexity of PDT with interactions between photosensitizer, light, oxygen and tissue pathophysiology. Many clinical applications of PDT have not been optimized due to the lack of detailed tumor and normal tissue photosensitizer and light dosimetry information. Although some empirically developed PDT treatments have been effective[24], it is important to continue to develop tools which allow objective assessment of the dosimetry, to optimize the treatment design and patient-specific planning.

1.2 Dosimetry in Photodynamic Therapy

The photodynamic dosimetry models that have been developed here are based on the assumption that direct tissue cell death mediated by $^{1}$O$_{2}$ is the primary determinant of the therapy outcome. There is a threshold dose of $^{1}$O$_{2}$ in the dosimetry model[25, 26]. This threshold dose refers to the minimum cytotoxic concentration of $^{1}$O$_{2}$. Cells receiving concentration of $^{1}$O$_{2}$ equal to or higher than the threshold dose will be damaged irreparably by the treatment, while those receiving $^{1}$O$_{2}$ less than the threshold dose will be able to survive treatment.

Initially, it was assumed that the concentration of $^{1}$O$_{2}$ is proportional to the product of the administered photosensitizer concentration and the total delivered optical density or fluence. This simplified dosimetry model was found to be valid only for a limited range of drug and light doses[1]. This is not surprising, since one of the basic
assumptions for such a model is that photosensitizer and oxygen availability as well as light delivery remains constant throughout treatment.

Studies demonstrating the variability in time and/or space of $^3$O$_2$ concentration, photosensitizer availability and light deliver to the treatment area during typical therapy protocols suggest that the dynamic character of these quantities should be incorporated in PDT dosimetry. In our dosimetry model, we assumed that knowledge of real-time local photosensitizer concentration and local light fluence will improve the accuracy of photodynamic dose calculation. Those dynamic factors result in different $^1$O$_2$ productions in the treated area, which correlate with differences in photodynamic therapy response.

Photosensitizer photobleaching could play an important role in modifying the effects of photodynamic oxygen consumption because irreversible destruction of the photosensitizer must reduce the photon absorption rate. More importantly, the photochemical destruction of photosensitizer also consumes local oxygen. It would be ideal to have $^1$O$_2$ as a direct parameter in PDT dosimetry estimation, since $^1$O$_2$ is the major cytotoxic factor relates to PDT response. But unfortunately, it is difficult to monitor changes in oxygen, light and photosensitizer simultaneously during PDT treatment. Photosensitizer photobleaching could be an alternative, implicit method for determination of the biological response that a specific treatment protocol could induce.

Furthermore, the interdependence of light fluence, photosensitizer concentration and oxygen consumption contribute to a very complicated scenario for the definition of photodynamic dose. In this study, an in vivo fluorescence dosimeter was used to monitor
the real-time photosensitizer and light fluence kinetic during 5-Aminolevulinic Acid - Protoporphyrin IX photodynamic therapy in a rat esophagus model. A photosensitizer photobleaching model was developed and applied in dosimetry estimation to improve the definition of the PDT dose.

1.3 5-Aminolevulinic Acid - Protoporphyrin IX Photodynamic Therapy for Ablation of Barrett’s Esophagus

Barrett’s esophagus is a major risk factor for the development of esophageal adenocarcinoma, approximately 15% of Barrett’s esophagi developed into esophagus cancer, and it is increasing in incidence in the Western world. Barrett’s epithelial metaplasia probably arises from duodeno/gastroesophageal reflux. Anti-reflux medications such as proton-pump inhibitors, H2 receptor blockers, or eventually anti-reflux surgery are the current treatment options. New forms of therapy are under investigation to completely eliminate Barrett’s epithelium and PDT is one of the techniques to destroy the abnormal mucosa and allow functional squamous regeneration [27]. PDT research has produced some viable clinical options within the past decade[22], and it appears to be an effective tool for ablating dysplasia and superficial cancers along with Barrett’s esophagus[27-33]. Photofrin and 5-Aminolevulinic acid (ALA) induced Protoporphyrin IX (PpIX) are effective photosensitizers for the PDT treatment of Barrett’s esophagus[34, 35].
In the first step of the heme biosynthetic pathway, 5-aminolevulinic acid (ALA) is formed from glycine and succinyl coenzyme A (CoA). The last step is the incorporation of iron into Protoporphyrin IX (PpIX), which takes place in the mitochondria under the action of the enzyme ferrochelatase. With the addition of exogenous ALA, PpIX may accumulate because of the limited capacity of ferrochelatase. Porphobilinogen deaminase is another enzyme of the heme synthesis pathway, which catalyzes the formation of uroporphyrinogen from porphobilinogen. In Barrett’s esophagus, there is an imbalance of porphobilinogen deaminase activity with that of ferrochelatase, so that PpIX accumulates almost entirely in the mucosa, rather than in the submucosa or muscularis mucosa.

In the accepted dosimetry model, photodynamic therapy efficacy depends upon the photosensitizer concentration. Measurement of photosensitizer uptake will help quantify the mechanisms of PDT damage and possibly optimize the treatment efficacy. The use of photosensitizer fluorescence has now become a major area of study in the detection and treatment of some tumors [36-41]. Using fluorescence to monitor pharmacokinetics has been under study for many years[1, 42-47], however improved fiber probes are now capable of providing a linear response between photosensitizer concentration and detected signal [48].

Though some studies show ALA-induced PpIX kinetics in tissues[49-52], the situation in vivo is complicated by variations in photosensitizer concentration and localization in tissue. In our previous study of aluminum phthalocyanine disulphonated pharmacokinetics, the results showed that photosensitizer uptake by the tumor can vary
significantly with tumor size, and even with the same tumor size, the variation is high between animals[1]. In order to help interpret the variability observed, and reduce dosimetry errors, a real-time photosensitizer concentration measurement tool was needed to investigate the photosensitizer concentration in tissue and carry out the PDT treatment according to the photosensitizer concentration, instead of simply the time between photosensitizer injection and PDT treatment.

The photobleaching of ALA-induced PpIX is also reported [20, 53, 54], yet its effect on PDT is not very clear[55, 56]. We assume that the photobleaching would reduce the PDT efficacy by reducing the PpIX concentration and hence the tissue singlet oxygen concentration during the PDT treatment. The effect of the photobleaching of PpIX on the PDT response is complicated. The effect of fractionation of the PDT treatment is suspected to be useful but still not well validated in the esophagus. Using a real-time PpIX dosimeter, we can monitor the PpIX concentration during the PDT treatment and can investigate the relationship of efficacy relative to the laser fluence rate, PpIX photobleaching kinetics and photobleaching.

1.4 Hypotheses

Based on our previous work in photosensitizer kinetics study and concerns about the complexity of dosimetry model in photodynamic therapy, several hypotheses were investigated as part of this research work:
Hypothesis 1: An animal model of Barrett’s esophagus could be developed by surgical techniques, and could be used for our pre-clinical ALA induced PpIX photodynamic therapy studies.

Hypothesis 2: A passive real-time in vivo dosimeter system for the esophagus could be used safely to monitor the both accurate photosensitizer and light fluence rate kinetics during PDT treatment of the esophagus.

Hypothesis 3: The Protoporphyrin IX distribution in the esophagus varies between subjects and is spatially heterogeneous for even a fixed administration pattern. A real-time dosimeter is necessary to measure individual subjects in real-time to improve dosimetry model accuracy.

Hypothesis 4: Protoporphyrin IX photobleaches rapidly during treatment and this affects treatment efficacy. The PpIX photobleaching kinetics also contains information on photosensitizer, light fluence rate and tissue oxygen (singlet/triplet) concentration. Interpreting PpIX photobleaching kinetics could help us improve our dosimetry model.

To verify these hypotheses, we developed a preclinical Barrett’s esophagus model and an in vivo dosimeter system, investigated Protoporphyrin IX pharmacokinetics and photobleaching during photodynamic therapy, and finally refined a photodynamic therapy dosimetry model to help us optimize treatment planning and improve future treatment efficacy in human clinical trials.

Overall, PDT dosimetry is a complicated function of photosensitizer
concentration, localization, light dose and tissue oxygen. The PDT treatment plan can not be simply designed according to non-biological parameters such as the light irradiation dose, photosensitizer injection dose and the time between injection and light treatment. The application of the passive PpIX dosimeter system developed here, to monitor the PpIX concentration in vivo during treatment, will help us understand the mechanisms of PDT treatment and how to optimize it currently with treatment for each patient separately. The models of photobleaching will also help us to determine the effective PDT parameters. The system is utilized in experimental animal model studies as a preliminary scientific analysis of how to optimize the therapy prior to eventual human studies.
Chapter II  Rat Model for Barrett’s Esophagus

In order to investigate dosimetry of photodynamic therapy for Barrett’s esophagus, a rodent animal model simulating the condition of Barrett’s esophagus was established and studied using a surgical intervention to irritate the esophagus. In this chapter, the characteristics of Barrett’s esophagus are examined and factors causing the condition are discussed, additionally a rat Barrett’s esophagus model was established to simulate the pathological presentation of the esophageal mucosa.

2.1 Definition of Barrett’s Esophagus

Adenocarcinoma of the esophagus is the most rapidly increasing incidence of solid tumor in the western world. A significant amount of research has therefore been focused towards pre-malignant conditions of the esophagus, such as Barrett’s esophagus, which are suspected to be associated with adenocarcinoma incidence.

Norman Barrett first described the esophageal condition as a segment of columnar-lined epithelium below a congenitally short esophagus in 1950[57]. Barrett described red glandular mucosa in the distal esophagus of some of his patients that he assumed to be a portion of the stomach which had been pulled into the distal esophagus as a result of the scarring following ulceration. Based on the early macroscopic definition, Barrett’s esophagus was mostly considered as a congenital condition rather than an
acquired condition[58].

As Barrett’s esophagus became the object of histopathologic and additional clinical studies, as it began to be considered that it could be acquired and that it was not necessarily composed of a gastric epithelium. Three types of glandular epithelium were identified as being characteristic of Barrett’s esophagus: specialized intestinal epithelium with goblet cells, junctional epithelium or cardia-antral type (without goblet cells), and fundic or oxyntic epithelium (also without goblet cells)[59]. In retrospect, the identification of these three types of epithelium may have been due to uncertainly about where the biopsies had come from, i.e., from the esophagus or from a hiatal hernia. The diagnosis of Barrett’s esophagus was thus based on two criteria: the histologic identification of one of those three types of epithelium and the finding of a segment with columnar metaplasia in the esophagus of at least 3-5 cm length[60]. Short segment Barrett’s was later defined as 2-3 cm in length, with Barrett’s epithelium <2 cm in length considered to be a normal variant. Pathology reports based on this concept identified the type of glandular epithelium that was present and used phrases such as “consistent with Barrett’s esophagus”, because the diagnosis was not based on histopathology alone.

The term “Barrett’s esophagus” has become firmly established at present, which began with the increasingly common use of endoscopy and the recognition that specialized columnar epithelium was not a normal finding in the distal esophagus, and that only this type of epithelium was associated with an increased risk of the development of adenocarcinoma[61]. It has been reported that a portion of patients with normal
endoscopic findings (regardless of indication for the endoscopy procedure) had specialized columnar epithelium in the vicinity of gastroesophageal junction[62-64]. Clearly this was not a normal finding. Because the term “Barrett’s esophagus” denotes a precancerous lesion, it was recommended that pathologists only use the term when specialized intestinal epithelium is found. Emerging consensus has shown that the diagnosis of Barrett’s esophagus is increasingly based on histopathologic findings alone, regardless of the length of the columnar epithelium.

Today, the definition of Barrett’s esophagus has become simple and general: The replacement of any portion of the normal squamous esophageal lining by a metaplastic columnar epithelium.

2.1.1 Characteristics of Barrett’s Esophagus

*Esophagus epithelium changes in Gastroesophageal reflux disease*

Based on epidemiologic and clinical studies, a clear link has been established between gastroesophageal reflux disease (GERD) and the development of esophageal specialized columnar epithelium.

The normal mucosa of the esophagus is composed of a stratified squamous epithelium resting upon a lamina propria. Changes consistent with reflux esophagitis include hyperplasia of the basal cell layer, broadening of the basal zone, extension of the papillae into the lamina propria, erosion and ulceration of the mucosa, and intraepithelial
eosinophils and neutrophils, which are less common. Mucosa capillary congestion and hemorrhage are also correlated with the signs of reddening seen by endoscopy. However, none of these changes are diagnostic for GERD and must be carefully compared with endoscopic and clinical findings.

**Specialized Intestinal Metaplastic Columnar Epithelium**

Barrett’s epithelium is a form of metaplasia in which the normal squamous lining is replaced by a glandular lining of the specialized intestinal type. Although earlier it was believed that this epithelium was the result of upward migration of gastric epithelium, it is now generally accepted that it originates from a multipotential cell in the esophagus itself, which could be found in the basal layer of squamous lining or among the cells lining the ducts of submucosal glands. It has also been reported that in some cases of Barrett’s esophagus, a hybrid epithelium can be found which has the appearance of stratified squamous epithelium but expresses cytokeratins, which are characteristic of glandular epithelium[65]. The reason for the formation of the glandular epithelium is not known, but presumably this type is more resistant to damage resulting from gastrooesophageal reflux.

Histologically, this specialized intestinal metaplastic columnar epithelium is characterized by a columnar epithelium that contains several cell types. At the surface, goblet cells, blue columnar, clear columnar and enterocyte-like cells with brush borders are found. Scattered Paneth cells can also be seen rarely, and also mature enterocytes are
observed. Below the surface, coiled, mucus-type glands are present. Although Barrett’s epithelium is thought to form as a result of gastroesophageal reflux, there is seldom a marked inflammatory component. It is now generally agreed that the goblet cell is the key cell type for the identification of Barrett’s epithelium. Usually goblet cells are easy to identify in a routine H&E stain, but at times they are not easily seen and an Alcian blue stain at a pH of 2.5 is recommended to help make them more apparent[66].

Based on histopathologic findings and the type of mucin produced, intestinal metaplasia throughout the gastrointestinal tract can be classified as “complete” (type I) or “incomplete” (type II or type III)[67, 68]. In type I intestinal metaplasia, columnar cells that have the features of small intestine absorptive cells are present, together with goblet cells and significant numbers of Paneth cells. Incomplete intestinal metaplasia may be either of the gastric (type II) or colonic type (type III), in which the columnar cells seen along with the goblet cells resemble gastric foveolar or colonic absorptive cells, respectively. Paneth cells are only occasionally seen in incomplete intestinal metaplasia. The columnar epithelium characteristic of Barrett’s esophagus is classified as intestinal metaplasia of the incomplete type (type II and type III) and has been found to be associated with gastroesophageal reflux disease (GERD), in contrast to the complete form of intestinal metaplasia, usually seen in the stomach, which is associated with atrophic gastritis[68].

The histopathologic definition of Barrett’s esophagus in use today is increasingly based on the histopathologic identification of columnar epithelium with goblet cells in the
esophagus, regardless of its length.

### 2.1.2 Factors Induce Barrett’s Esophagus

It may be commonly accepted that GERD sets the scene for the development of Barrett’s esophagus, but there is still a lot of controversy about the role of the constituents of the reflux able to induce injury and subsequent metaplastic change at the gastroesophageal junction. In order to establish a better animal model of Barrett’s esophagus, it is necessary to investigate the major factors that will induce Barrett’s esophagus.

Gastroesophageal reflux certainly is not homogenous, but consists of a broad spectrum of alkaline, neutral and acid components: oro-esophageal (saliva, food, esophageal secretions), gastric (acid, mucus, pepsin), and duodenal (amylase, lipase, trypsin, chymotrypsin, alkaline salts, bilirubin, bile salts, and cholesterol) origin.

There is no doubt that reflux of acid into the esophageal lumen plays an important role in the pathogenesis of Barrett’s esophagus. Many reports confirm a more important esophageal exposure to acid in patients with Barrett’s esophagus compared with controls[69, 70].

Duodenal juice consists of a broad spectrum of components including pancreatic, duodenal, and biliary secretions. The presence of duodenal contents in the esophageal lumen has been linked to the development of mucosal injury and Barrett’s esophagus[71].
Esophageal mucosal injury by refluxed duodenal juice has been shown to depend on the conjugation state of bile acids as well as the pH of the refluxate. The combined reflux of gastric and duodenal juices causes severe esophageal mucosal damage. The vast majority of duodenal reflux occurs at a pH range of 4 to 7, at which bile acids, the major component of duodenal juice, are capable of damaging the esophageal mucosa[72].

2.2 In Vivo Rat model of Barrett’s Esophagus

Based on the knowledge of the factors inducing Barrett’s esophagus, an in vivo rat model was established. In the rat, reflux esophagitis develops when duodenal contents reflux into the esophagus. The pancreaticobiliary secretion is the injurious factor, pancreatic juice being less important than bile. In order to better induce Barrett’s esophagus, our model was established to generate reflux containing pancreaticobiliary secretions and some gastric secretion. As illustrated in Figure 2.1 and Figure 2.3 b, we select the anastomosis of the esophagus to jejunum at the position approximately 3 cm post duodenum, and leave the stomach as an end pouch after the transaction of esophageal-gastro junction. Thus, both pancreaticobiliary and gastric secretions will have the possibility to reflux into the esophagus at the anastomosis junction.

Materials and Methods

The Dartmouth College Institutional Animal Care Committee (IACUC) approved
the animal study. Six-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and housed 3 per cage under standard laboratory conditions (room temperature, 22 ± 2 °C; relative humidity, 55% ± 5% and 12-hour light/dark cycle). Rats were given commercial rat chow before and after surgical intervention. The rats were allowed to acclimate for 2 weeks before surgical intervention. A total of 24 rats were operated on, and an additional 5 rats were followed without any intervention to obtain normal esophageal tissue. Rat chow was withheld 48 hours before surgical intervention, and liquids were stopped on the morning of surgery. General anesthesia was induced by isoflurane (1.5% isoflurane with 98.5% O₂) and maintained throughout the surgery.

Levrat’s esophagojejunostomy technique was used in this approach[73]. As illustrated in Figure 2.1 and 2.2, a midline laparotomy was performed, and the gastroesophageal junction was identified and carefully isolated from nerve and blood vessels. The gastroesophageal junction was ligated, and the distal esophagus was transected 2 mm above the ligature. The end of the esophagus was preserved carefully during the surgery. A 5 mm jejunostomy was completed on the jejunum approximately 3 cm posterior to the duodenum. A total of 8 polypropylene 7-0 sutures were placed along the transaction of end of esophagus. An end-to-side esophagojejunostomy was completed as illustrated in Figure 2.1 and 2.2.
Figure 2.1 illustrates the anatomical changes following the esophagojejunostomy surgery. (a) is the anatomy of normal rat upper GI track, esophagus connected to stomach. (b) esophagus connected to jejunum approximately 3 cm posterior to the duodenum.
Figure 2.2 Surgical creation of a rat model of Barrett’s esophagus. (a) The gastroesophageal junction (white arrow) was identified and isolated for esophagus transection. (b) The vagus trunk (white arrow) was preserved during transaction. (c) A 5-mm jejunostomy was created approximately 3 cm post duodenum. (d) While carefully maintaining the orientation and patency of the lumen, an end-to-side esophagoenterostomy was performed.
The anastomosis area was placed between the lobes of liver. Topical analgesic (buprenorphine 50 µl of 0.1 mg/ml) was instilled in the peritoneal cavity, especially on the anastomosis area. The abdominal wall was closed in 2 layers with 5-0 monofilament. To compensate for hydration loss, 5 ml of sterile 0.9% NaCl was injected subcutaneously. Water was provided once the rats were awake, and rats were fed with the soft chow by the first post-surgery day and provided regular food 3 days later. Buprenorphine for post operation analgesia (50 µl of 0.1 mg/ml) was injected intramuscularly twice per day for 2 days post surgery. Rat weights were recorded up to 35 days post surgery. 11 rats with esophagojejunostomy surgery were kept alive for 25-30 weeks and been using in further research projects (detailed in chapter IV).
Figure 2.3 Esophagus and jejunum anastomosis of rat (57 days post surgery). (a) Anastomosis (white arrow) lateral with stomach, this figure illustrate the similar organ positions in rat body. (b) Anastomosis (white arrow) is located approximately 3 cm posterior to the duodenum (dark colored on the left). This anastomosis permitted both duodenal and gastric reflux into the esophagus.
2.3 Results and Analysis

2.3.1 Rats Health Analysis Post Esophagojejunostomy Surgery

A total of 24 Sprague-Dawley rats received esophagojejunostomy surgery. Three rats were used as pilot study and sacrificed by lethal injection of saturated KCl immediately following surgery. A total of 7 rats died within 2 days post surgery due to surgical complication. Three rats died at an early stage post surgery (11 days, 14 days and 15 days respectively), and 11 rats (52.4%) survived longer than 2 weeks post surgery. Procedure success rate is 40%. Three rats were sacrificed at days 57, 68 and 152 for histopathology study, and 8 rats were survived at least 30 weeks post surgery and were used for photodynamic analysis of Protoporphyrin IX production study (Chapter IV).

Rats were monitored daily at the first 2 weeks post surgery for signs of failed surgery, by assessing weight, food/water consumption, and mobility. Three rats considered as failed surgery (dead at day 11, 14 and 15) consumed less food daily (0-10 g regular rat chow), compared with the other 11 rats with successful surgery (15-25 g food consumed daily).
Figure 2.4 Rat weight changes post esophagojejunostomy surgery. Blue solid lines show the weight change of 11 survival rats, red dashed lines show the weight change of 3 dead rats with failed surgery (death date 11 14 and 15). There was significant weight drop at the first 2-5 days due to the fasting procedure 48 hr pre surgery and 24 hr post surgery. With the food and recovering from surgery, rats began to gain weight after 2 weeks post surgery. Rats regulated about 2 weeks to fully recover from surgery and begin to gain weight.

Rats’ weights were recorded every day for two weeks post surgery and every other day thereafter up to one month. As illustrated in Figure 2.4, there was significant weight drop at the first 2-5 days due to the fasting procedure 48 hr pre surgery and 24 hr post surgery. With the access to food and recovering from surgery, rats began to gain weight after 2 weeks post surgery. The red dash lines in the figure show the weight change of 3 rats considered as failed surgery, those 3 rats kept loosing weight and less active moving were also observed from daily monitoring. Be considered as successful
surgery, 11 rats show substantial food consumptions and weight increasing post surgery.

2.3.2 Pathology Study of Rats Esophagus Post Surgery

Three Rats were sacrificed by lethal injection of saturated KCl, at day 57, 68, 152 respectively following surgery. And eight Rats were sacrificed 30 weeks following surgery after PpIX kinetic study (Chapter IV). Esophagi were extracted for histopathology study. Histology analysis was studied on sections processed in the Histopathology Laboratory, Department of Pathology, Dartmouth Hitchcock Medical Center. Macroscopic image of the esophagus-jejunum anastomosis showed ulcerative and hyperplastic esophagitis (Figure 2.5 a). Columnar epithelium was observed in the area, microscopically (Figure 2.5b). The histological sections of the esophagus showed classic Barrett’s esophagus alteration as compared to the normal esophagus, which had regular stratified squamous epithelium (Figure 2.6 a and Figure 2.7 a).

Hyperplasia: The hyperplastic reaction was confined to the mucosa, causing it to become 3-4 times thicker than normal (Figure 2.6). This epithelial hyperplasia was associated with a hyperkeratosis (Figure 2.5b, 2.7b and c).

Ulceration: The ulceration in some instances was superficial, with only a superficial abrasion of the keratin layer of the epithelium. In other cases, there was disappearance of a sizeable portion of the epithelium (Figure 2.6c the upper portion and Figure 2.6d the left portion of epithelium), with the chorion dipping into the cavity
between swept epithelial crests. In some other instances, the ulcerations were characterized by total disappearance of all epithelial layers, the bottom of the cavity being formed by the chorion.

Figure 2.5 Esophagus epithelium changes 57 days post esophagojejunostomy surgery. (a) Macro view of esophagus anastomosed to jejunum shows ulcerative and hyperplastic esophagitis at the lower esophagus. (b) Microscopy sections shows columnar epithelium was formed at the lower esophagus.
Figure 2.6 4× microscopy sections of esophagus with H&E stain. (a) Normal rat esophagus. (b) esophagus extracted from rat at 68 days post surgery. Compared to the normal esophagus, the hyperplastic reaction was confined to the mucosa, causing it to become 3-4 times larger than normal. Ulceration was observed (the upper portion in figure d).
**Inflammation:** Polymorphonuclear and lymphocytic infiltration were found in the mucosa and submucosa with capillary budding.

**Dysplasia:** Dysplasia was found in all three cases, characterized by hyperchromatic, enlarged nuclei and depletion of cytoplasmic mucin (Figure 2.7 b c and d). The process often extends to the surface of epithelium with architectural changes that include budding of glands, and pseudo-stratification.

![Figure 2.7 40× microscopy sections of esophagus with H&E stain.](image)

(a) Normal rat esophagus. (b) 57 days post surgery. (c) 68 days post surgery. (d) 152 days post surgery. Compared to normal esophagus with neat even epithelium, esophagi with anastomosis to jejunum show columnar disorder epithelium layers. Dysplasia was found in all three cases, characterized by hyperchromatic, enlarged nuclei and depletion of cytoplasmic mucin. Inflammatory cells were also observed.
Figure 2.8 40× microscopy sections of esophagus with H&E stain. (a) published illustration of human Barrett’s esophagus section (www.barrettsinfo.com). (b)-(f) rat esophagus sections extracted 30 weeks post surgery. Columnar disorder epithelium layers and dysplasia were found in most cases (b–e), characterized by hyperchromatic, enlarged nuclei and depletion of cytoplasmic mucin. (f) Barrett’s esophagus was not seen in this esophagus section. The epithelium was not altered.
Compared to published human Barrett’s esophagus histopathology microscopic image (Figure 2.8 a), most rat esophagi with surgery (7 out of 8) treated rats developed Barrett’s esophagus changes such as columnar disorder epithelium layers, dysplasia, enlarged nuclei and glandular tissues (Figure 2.8 b-e).

2.4 Conclusions

In conclusion, our study suggests that both duodenal and gastric secretions are involved in the development of Barrett’s esophagus. By the esophagojejunostomy surgery, both duodenal and gastric secretions were refluxed into the esophagus thru anastomosis. Our studies showed that rats received successful esophagojejunostomy surgery could survive long enough (30 weeks) to demonstrate classic Barrett’s esophagus. Epithelial changes were observed as early as 60 days. The changes were characterized as hyperplasia, ulceration, inflammation and dysplasia. The columnar epithelium was easily observed in macroscopy. Thus, a rat Barrett’s esophagus model has been successfully established and it is ready to be used in photodynamic studies.
Chapter III  Development of a Passive Dosimetry System for the Esophagus

3.1 Fluorescence Measurement of 5-Aminolevulinic Acid Induced Protoporphyrin IX

In recent years, 5-Aminolevulinic Acid (ALA) has increased in usage as a pro-drug for photosensitization [74, 75]. ALA is metabolized within tissue to form the photosensitizer molecule Protoporphyrin IX (PpIX), and owes its application to the selectivity with which Protoporphyrin IX is formed within cancerous tissue, and the ease of use of ALA itself.

An important factor in photodynamic therapy dosimetry, is the necessity to develop an *in vivo*, non-invasive method to quantify PpIX concentrations in real-time. Like most photosensitizer, PpIX is mildly fluorescent, as shown in Figure 3.1, it has a small Q-band absorption peak at 633 nm. When excited by visible light, PpIX will produce fluorescence with a peak around 645 nm and with a secondary peak near 700 nm. This latter peak is most useful since it is able to be filtered from the excitation wavelength light at 635 nm (Figure 3.1). Measuring fluorescence from PpIX is a feasible method for dosimetric measurement of the quantity [52, 53, 76], and in this work, a small fiber-optic based system was used to produce real-time ‘microsampling’ of
photosensitizer tissue fluorescence[1, 48, 77].

Figure 3.1 The emission spectrum (excited at 635 nm) of Protoporphyrin IX (PpIX) is shown in saline, as acquired by a cuvette-based spectrophotometer (SPEX FluoroMax-3). The solid lines are the PpIX spectrum before PDT irradiation. Dashed lines are fluorescence (may include photoproduct from PpIX photobleaching) spectrum after 10 J/cm² PDT irradiation. PpIX photobleaching product has a fluorescence peak around 670 nm while PpIX itself has a fluorescence peak around 700 nm.

Figure 3.1 also shows the spectrum of PpIX after 10J/cm² irradiation to the 1 cm cuvette solution. It is well known that PpIX is photobleached quickly [20, 78]. The change of the spectrum after 633 nm laser irradiation indicates the existence of the photobleaching products, which has a peak near 670 nm. Choosing an appropriate filter to eliminate this peak helps to make sure the detected peak signal is truly reflective of the
PpIX concentration.

In order to noninvasively measure PpIX in real-time for photodynamic therapy of Barrett’s esophagus, a passive esophagus dosimetry system was developed. In this chapter, we describe the system setup and calibration studies completed. And this system will be applied in the Protoporphyrin IX kinetic study (Chapter IV) and PDT treatment for esophagus (Chapter V). With real-time quantitative information of the fluorescence, it is possible to better understand the mechanisms of photosensitizer photobleaching and its impact upon the treatment. Finally the dosimeter could ideally be used in real time to provide feedback to improve individual PDT treatment.

### 3.2 Description of a Passive Esophagus Dosimetry System

An esophageal dosimetry system (Figure 3.2, 3.3 and 3.4) was successfully constructed, to passively measure the porphyrin fluorescence (i.e. concentration) during PDT treatment. The dosimeter has 6 input fibers from 6 different sites of the esophagus (Figure 3.4). In this “passive” design, the fluorescent signals excited by the treatment laser (633nm) and would be acquired by the fiber tips which are attached to the treatment balloon. The optical signals that contain porphyrin fluorescence signal (fluorescence channel measurement) and the treatment laser signal (reference channel measurement) are separated by a splitter and then measured by two different PMTs (Figure 3.3 d). These signals from different sites are recorded sequentially in a rapid cycling manner.
According to the PpIX fluorescence spectrum excited by a 633nm laser (example spectrum shown in Figure 3.1 and 3.3 c), two filters (620nm-650nm band-pass filter for the reference channel and 690nm high-pass filter for the fluorescence channel) are placed before the PMTs to reduce cross-talk between the two optical channel signals and eliminate the noise. A LabView program has been written to control the whole process.

**Excitation laser**

Protoporphyrin IX fluorescence signal was excited by the Photodynamic therapy treatment laser (635nm) using 2 different lengths of diffused fiber tips. For large animal (i.e. pig) studies or human clinical trials, the treatment laser would irradiate tissue from a 5 cm long diffused end fiber tip (Figure 3.4 c), while a 1 cm long diffused end fiber tip was used for small animal study (i.e. rat) (Figure 3.4 d)
Figure 3.3: The overview and key components of the dosimetry system are shown. (a): The dosimetry system is comprised of the 6 to 1 optical switch (upper-left), signal detector (lower-left), electronic control board (upper-middle) and DAQ card (lower-right). (b): The 6 to 1 optical switcher will receive 6 input optical signals and transfer these signals to the detection part sequentially. (c): The fluorescence spectrum of PpIX excited by 635nm. The PpIX has a fluorescence peak around 700nm, and this fluorescence peak will be used in our dosimetry system to measure the PpIX concentration. (d): The detection part includes two PMTs and two sets of filters, the optical signal will be divided into two channels and give us the information about the PDT irradiation signal and PpIX fluorescence signal.
Figure 3.4 (a) The MR image for the pig esophagus and the balloon probe is shown. In (b) the esophagus with the dosimetry balloon probe is shown, with (c) the dosimetry balloon probe for the pig esophagus, and (d) the dosimetry probe used for the rat esophagus.

_Emission sensor_

Both the PpIX fluorescence signal and the PDT treatment laser signal were acquired by diffused end tip fibers. The diffused end tip is 1 cm long with the diameter of 200 microns. Two different kinds of probes were used for signal acquisition. For large animal studies, a balloon probe was used (Figure 3.4 a b and c) with 6 detection fibers attached to the balloon membrane at 6 different positions. After the probe was inserted into esophagus and reached the treatment area, the balloon probe was inflated and 6
detection fibers were closely attached to the esophagus, thus those fibers would acquire both signal intensity and signal spatial information. For small animal studies, a single detection fiber was attached right beside the PDT irradiation fiber (Figure 3.4 d).

*The 6 to 1 mechanical fiber switcher*

After the detection diffused end tips, 6 fibers were lined to a 6 to 1 fiber coupler. Right before the coupler, 6 shutter sets were applied (Figure 3.3 b). Each shutter set consisted of a mechanic shutter, 2 collimators and an aluminum holder. The shutter was controlled by Labview to open/block the optic path between 2 collimators. Each of the 6 input fibers of the coupler were 200 microns and the output fiber was 800 microns which is lined to the optical collimator (Figure 3.3 d). By sequentially open and close each shutter, the signal from 6 different diction fibers are sent to the PMT via this single optical pathway.

*Optical setup*

The optical signal from the detection fiber contained both PDT treatment laser information (around 635 nm) and Protoporphyrin IX fluorescence information. It also contained information of the photoproducts of Protoporphyrin IX, as shown in Figure 3.1. The photoproduce of Protoporphyrin IX (most likely the result of Protoporphyrin IX photobleaching) yields fluorescence with a peak around 670 nm of 10 nm bandwidth. It is important to separate these two signals. As illustrated in Figure 3.2 and Figure 3.3 d, the
photons from the detection fiber hit a dichroic mirror and are divided into two difference channels. One channel is designed as <reference channel> with a 620-650 nm band pass filter right before PMT. This reference channel is for PDT treatment laser (635 nm) intensity measurement. Another channel is designed as <fluorescence channel> with a 690 nm longer pass filter right before PMT. This fluorescence channel is for PpIX fluorescence measurement, and the photoproduc fluorescence photons were blocked by the filter. The readings from the fluorescence channel are believed to be mostly PpIX fluorescence intensity.

**PMT setup**

Separate photomultipliers (PMT, HAMAMATSU HC120) were used to measure the optical signals in each of the two channels. The PMT gains were fixed at 0.6 Volts and 0.8 Volts for reference and fluorescence channel respectively, were the input range is from 0 to 1.0 Volts. The signals coming from PMTs are acquired by data acquisition card (National Instruments DAQ 2064E) which is driven by Labview.

**Labview program setup**

A Labview program was developed for the dosimetry system control, data acquisition and result display. For each measurement, signal intensities (PMT voltage) of each channel were recorded along with detection fiber index number, PMT gain values and measurement comments. User interface was illustrated in Figure 3.5.
3.3 System Calibration in Liquid Phantom

As the dosimeter system was constructed, a series of calibration studies in liquid phantoms were designed to verify the consistency of the dosimeter, and to investigate the correlation between the PDT treatment laser output power and reference channel signal, and also to investigate the correlation between Protoporphyrin IX concentration and fluorescence channel signals.
Liquid phantoms were made up by different concentrations of Intralipid, ink and Protoporphyrin IX. A mix of 1% Intralipid approximately equals the reduced scattering coefficient of $\mu_s' = 1.1 \text{ mm}^{-1}$, with 1% ink approximately equal to absorption coefficient of $\mu_a = 0.025 \text{ mm}^{-1}$ at 635 nm. To simulate the measurement in esophageal tissue, phantoms were made to approximately the same optical properties as $\mu_s' = 1.1 \text{ mm}^{-1}$ and $\mu_a = 0.04 \text{ mm}^{-1}$, which is thought to be representative of this tissue. Liquid phantoms were held in 15 ml plastic tubes with a total volume of 10 ml.

Figure 3.6 Correlation of reference channel signals to the laser output is shown. Measurements were taken in liquid phantoms with the similar optical properties as $\mu_s' = 1.1 \text{ mm}^{-1}$ and $\mu_a = 0.04 \text{ mm}^{-1}$. No Protoporphyrin IX was added in phantoms. Laser output changes from 0 mW/cm to 165 mW/cm. The reference channel PMT output signals (voltage) has linear correlation with laser outputs.
3.3.1 Light Dosimeter Calibration

A total of 3 sets of liquid phantoms were made with $\mu_s'=1.1\text{mm}^{-1}$ and $\mu_a=0.04\text{mm}^{-1}$, but no PpIX was added in phantoms. The detection fiber was inserted into the center of the liquid. Laser outputs were set at 0, 14, 21, 32, 67, 128 and 165 mW/cm, and measurements were taken at each of these levels.

As illustrated in Figure 3.5, the reference channel PMT output signals (voltage) had linear correlation with laser outputs. Since 25 mW/cm, 50 mW/cm and 100 mW/cm are the most using laser powers for ALA-PpIX PDT treatment in the esophagus, our phantom study demonstrated a reliable laser dosimeter in our system. The reading from the reference channel PMT could be used to calculate the PDT light dose.

3.3.2 Correcting for Signal Cross-talk

From the light dosimeter calibration phantom study, it was also noticed that even without any PpIX inside the phantoms, there still were some readings from the fluorescence channel PMT. This was concluded to be crosstalk from the irradiation laser, assuming the 690 longer pass filter could not block all photons in the range of 620-690 nm. To investigate the crosstalk between reference channel and fluorescence channel, a new set of phantom studies was designed.
A total of 4 sets of phantoms were made. Considering there could be some optical variations in individual targets, absorption coefficient $\mu_a$ was different in each phantom ($\mu_a = 0.0125, 0.025, 0.04$ and $0.05 \text{mm}^{-1}$). Reduced scattering coefficient $\mu_s$ was still set at $1.1 \text{mm}^{-1}$ for all phantoms and no Protoporphyrin IX was added into the phantoms. Laser outputs were set at 32, 67, and 128 mW/cm, and measurements were taken at each of these settings.

As seen in Figure 3.6, the result showed that the cross-talks from the reference channel was linearly increasing with the laser output, yet the cross-talk was also very consistent as the optical properties varied within the pertinent range of possible values. This data was fitted into a linear function $y=kx+b$, where the reference channel ($x$) to fluorescence channel ($y$) crosstalk functions were: $y=0.108x+0.068$ ($\mu_a = 0.0125 \text{mm}^{-1}$), $y=0.110x+0.062$ ($\mu_a = 0.025 \text{mm}^{-1}$), $y=0.112x+0.060$ ($\mu_a = 0.04 \text{mm}^{-1}$) and $y=0.118x+0.059$ ($\mu_a = 0.05 \text{mm}^{-1}$) respectively. The slope rate $k$ had 3.8% variation while $b$ has 6% variation, indicating that these calibration lines could work for a broad range of tissue optical properties.

With this consistent linear crosstalk function, readings from fluorescence channel could be adjusted according to reference channel readings and the crosstalk could be effectively corrected for.
Figure 3.7 Crosstalk from reference channel to fluorescence channel. Each solid line refers to an set of phantoms (same reduced scattering coefficient $\mu_s'=1.1 \text{ mm}^{-1}$ and different absorption coefficient $\mu_a = 0.0125, 0.025, 0.04$ and $0.05 \text{mm}^{-1}$). No PpIX was inside the phantoms, and the laser output changed from 32 mW/cm to 128 mW/cm. Within the expected ranges of absorption coefficient, the crosstalk remain consistent to within 6\% standard deviation.

### 3.3.3 Photosensitizer Dosimeter Calibration

In order to investigate the correlation between detected fluorescence signal and PpIX concentration, 8 sets of liquid phantoms were made with same optical properties ($\mu_s'=1.1\text{mm}^{-1}$ and $\mu_a=0.04\text{mm}^{-1}$). A total of 8 different concentrations of PpIX were added into phantoms (0, 0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0 ug/ml respectively). Irradiation laser output was set at 50mW/cm and 6 measurements were taken for each phantom.
Figure 3.8 Two channel PMTs Readings of liquid phantom with different Protoporphyrin IX concentrations. Measurements were taken in liquid phantoms with the similar optical properties as  μˈs'=1.1mm⁻¹ and  μs=0.04mm⁻¹. Protoporphyrin IX concentrations change from 0 ug/ml to 3 ug/ml. Blue dots are refer to fluorescence channel PMT readings, while red crosses are refer to reference channel PMT readings. The reference channel PMT reading did not show positive correlation with Protoporphyrin IX concentrations, which indicated a minor crosstalk effect of the fluorescence channel into the reference channel. The reference channel signal could be considered independent.

Two channel PMT readings are illustrated in Figure 3.7. The fluorescence channel readings had some positive correlation with PpIX concentration, while reference channel readings did not. Reference channel readings varied ±4% and decreased a little while the PpIX concentration (fluorescence intensity) increasing significantly. This minor decrease of reference channel signal was reasonable with the assumption that the PpIX would affect the phantom absorption coefficient somewhat. From the consistency of the
reference channel readings, it was assumed that the crosstalk from the fluorescence channel to the reference channel was a minor factor. Only crosstalk from reference channel to fluorescence channel need to be addressed.

![Graph showing correlation of fluorescence channel signals to PpIX concentrations](image)

**Figure 3.9** Correlation of fluorescence channel signals (after crosstalk adjustment) to PpIX concentrations. This figure illustrated a positive linear correlation between dosimeter system readings and Protoporphyrin IX concentrations in liquid phantom. The detection precision was 0.1 ug/ml Protoporphyrin IX, and the linear range is up to 4 ug/ml.

Using the crosstalk function \(y=0.112x+0.060\) to adjust the fluorescence channel readings, the result was shown in Figure 3.8. A linear relationship between the fluorescence channel signal (after crosstalk adjustment) and the Protoporphyrin IX
concentrations was observed. It was encouraging that this passive dosimetry system could
detect down to 0.1µg/ml difference in the Protoporphyrin IX concentrations, while the
linear range included 0 to 4 µg/ml, which was also the in vivo Protoporphyrin IX
concentration range expected in tissue.

In summary, a passively monitoring in vivo fluorescence dosimeter was
successfully developed to measure dosimetry parameters during the PDT treatment. The
calibration studies in liquid phantoms showed the feasibility of applying the dosimeter
into future PDT dosimetry studies, including light dose measurement and in vivo with
simultaneous PpIX measurement.
Chapter IV  5-Aminolevulinic Acid Induced Protoporphyrin IX Pharmacokinetics in Normal/Barrett’s Rat Esophagus

4.1 Introduction of 5-Aminolevulinic Acid Induced Protoporphyrin IX

5-aminolevulinic acid (ALA) is an endogenous compound that is part of the heme biosynthetic pathway. As illustrated in Figure 4.1, in the first step of the heme biosynthetic pathway, ALA is formed from succinyl coenzyme A (CoA) and glycine. The last step is the incorporation of iron into Protoporphyrin IX (PpIX), which takes place in the mitochondria under the action of the enzyme ferrochelatase (“A” marked in Figure 4.1). With the addition of exogenous ALA, PpIX may accumulate because of the limited capacity of ferrochelatase. Porphobilinogen deaminase (PBG-D) is another enzyme of the heme synthesis pathway (“b” marked in Figure 4.1), which catalyzes the formation of uroporphyrinogen from porphobilinogen (PBG). ALA itself has no photosensitizing properties but it is converted via a variety of other porphyrins to Protoporphyrin IX (PpIX), which is photosensitizer.

In Barrett’s esophagus, there is an imbalance of porphobilinogen deaminase (PBG-D) activity with that of ferrochelatase (FC), therefore leading to greater production of Protoporphyrin IX, the photosensitizer[79].
In contrast to other photosensitizers, PpIX accumulates almost entirely in the mucosa, rather than in the submucosa or muscularis mucosa[80]. This allows for a treatment penetration that is ample to destroy the metaplastic columnar epithelium, which approaches 0.5 mm in depth[81].

Figure 4.1 The heme biosynthetic pathway is illustrated schematically (A, Ferrochelatase with Fe2+; B, PBG-deaminase).

ALA-PpIX based PDT has been applied in several clinical trials. The first clinical trial of ALA-PpIX PDT in patients with Barrett’s esophagus was reported in 1996 by Barr et al[82]. Ortner et al treated 14 patients with Barrett’s esophagus with topical ALA administration by spraying ALA onto the mucosa at a dose of 14-16 mg/kg[83]. The procedure was tolerated well, with mild retrosternal chest pain and minimal dysphagia but no systemic side-effects[84]. Other investigators have reported clinical trial results of
PDT with oral ALA administration[85, 86], yet unpublished reports indicate that high oral doses of ALA are not well tolerated and lead to high levels of nausea and vomiting which are the dose limiting factor of morbidity.

In a simple dosimetry model, photodynamic therapy efficacy depends upon the photosensitizer concentration. Measurement of photosensitizer uptake will help quantify the mechanisms of PDT damage and possibly optimize the treatment efficacy. The use of photosensitizer fluorescence has now become a major area of study in the detection and treatment of some tumors [36-41], as a way to best predict the treatment effect or plan the treatment. Using fluorescence to monitor pharmacokinetics has been under study for many years[1, 42-47], and several optimal system designs are known.

Although some investigators have clearly studied the ALA-induced PpIX kinetics in tissue[49-52, 87], the situation in vivo is still too complex to fully interpret, because of the variation of the photosensitizer concentration and localization in tissue and changes with time and route of administration. In this chapter, several methods are used to measure ALA-PpIX kinetics in rat normal esophagus and the model Barrett’s esophagus, and the PpIX uptake differences between systemic and topical administration of ALA are compared.
4.2 ALA-PpIX Pharmacokinetics in Rat Normal Esophagus

4.2.1 Materials and Methods

In this study, the focus is on the PpIX uptake at different time points post ALA i.p. administration and the goal was to find out the conditions at which optimal PpIX uptake was observed in the esophagus. This would help design future PDT treatment conditions to the optimal time point and method of application.

Animal

The Dartmouth College Institutional Animal Care Committee (IACUC) approved this animal study. Six-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and housed 3 per cage under standard laboratory conditions (room temperature, 22 ± 2 °C; relative humidity, 55% ± 5% and 12-hour light/dark cycle). The rats were allowed to acclimate for 2 weeks before surgical intervention. Rat chow was withheld 24 hours before surgical intervention, and liquids were stopped on the morning of PDT treatment.

Photosensitizer and quantification

The ALA was used as a photosensitizer in all studies; it was purchased from Sigma Inc. and prepared in a stock solution at 60 mg/ml in sterile PBS. The stock solution of ALA was kept in the dark at 4°C to prevent photobleaching.
Three methods to quantify the PpIX uptake in the esophagus tissue and plasma were used, including *in vivo* fluorescence system and *ex vivo* fluorescence scanner system in esophageal tissue; and an *ex vivo* fluorescence spectrometer system for PpIX in plasma. The *in vivo* method used the passive dosimeter described in Chapter III.

*Experiment setup*

A total of 23 normal Sprague-Dawley rats were used in this study. Animals were randomized into 4 groups according to different incubation times (2hr: n=6, 4hr: n=6 and 24hr: n=6) and intraperitoneally (i.p.) administrated with photosensitizer (ALA) at 100 mg/kg, and rats in the control group (n=5) were i.p. administrated with saline. At different time points, our passive fluorescence dosimeter was put into the rat esophagus, so that the *in vivo* PpIX fluorescence could be measured. During the same time, samples of 2 ml of the rat blood were acquired from the heart. Immediately after the blood extraction, each rat was sacrificed by lethal injection of saturated KCl. The esophagus was extracted and opened for *ex vivo* fluorescence scanner measurement (Typhoon™ 9410 by GE) to quantify the signal from the entire esophagus. The blood sample was centrifuged with a speed of 2500 RPH at 4 °C for 5 minutes. The plasma was extracted for fluorescence assessment via a cuvette-based spectrophotometer (SPEX FluoroMax-3). The measurement of rats in the control group was taken at the 2 hr time point.
4.2.2 Results and Analysis

Both \textit{in vivo} and \textit{ex vivo} measurements of PpIX fluorescence in the esophagus yielded similar PpIX uptake kinetics (illustrated in Figure 4.2). From the in vivo dosimeter measurement, the control group showed a fluorescence ratio of background/endogenous of 1.04 ± 0.78 ug/ml. At 2 hours after ALA i.p. administration, the PpIX increased significantly (p-value=0.0004) to a level of 3.26 ± 0.32 ug/ml. After 4 hours, the PpIX signal in the esophagus decreased (2.06 ± 0.87 ug/ml at 4hr) and return to an equivalent to background level at 24 hours (1.10 ± 0.80 ug/ml). Large standard deviation values indicate significant variations exist between individual animals. From the ex vivo fluorescence scanner measurement (with a total fluorescence intensity scale of 256), the PpIX fluorescence increased significantly (P-VALUE=0.0002) from 73.7 ± 5.0 to 101.0 ± 7.5 at 2 hours post ALA administration. Similar to the in vivo situation, PpIX fluorescence also decreased to background levels at 24 hours (63.2 ± 9.3).
Figure 4.2 PpIX fluorescence signal in normal rat esophagus tissue as measured in situ by two methods: the in vivo fluorescence dosimeter (a) and the ex vivo fluorescence scanner (b). Both methods yield similar PpIX uptake kinetic. PpIX in the esophagus has an uptake peak at early hours post ALA administration and drops back to background level at 24 hour (* p-value=0.0004, # p-value=0.0002). (Animal used in control: n=5; 2hr: n=6; 4hr: n=6; 24hr: n=6).

PpIX fluorescence was also measured in the plasma with an *ex vivo* spectrometer. The result is shown in Figure 4.3. The PpIX fluorescence in the plasma increased over time as it was produced in the cells and it diffused into blood, as it is known to do in vivo.
The signal from the blood stayed at a high level throughout the 2 to 24 hour period. The mechanism of ALA-PpIX systemic distribution is still unclear from this data, although it is believed to simply diffuse out of the cells that created it. The result of low PpIX levels in esophagus and high level in plasma at 24 hours may indicate that there are several other tissues had high PpIX uptake/productivity as well, leading to continuous diffusion of it to the blood, or more simply a lack of effective clearance of it from the blood within the 24 hour period. Further study was carried out to isolate exogenous ALA in the esophagus and compare to PpIX productivities from other tissues.

Figure 4.3 PpIX signals measured from plasma measured in the spectrometer. The PpIX fluorescence in the plasma increased after the ALA administration and stayed at a high level for to the duration of the 24 hour period studied here. (Animal used in control: n=5; 2hr: n=6; 4hr: n=6; 24hr: n=6).
4.3 ALA administration Methods in Normal/Barrett’s Rat Esophagus:

Topical vs. Systematical Administration

In this next phase of the study, the focus was on PpIX signal variation with different ALA administration methods, and how this would be used in the rat Barrett’s esophagus model.

4.3.1 Materials and Methods

A total of 27 Sprague-Dawley rats were used in this study, with 20 rats having normal esophagus, and randomized into 3 groups.

Group A was a control group without any ALA administration (n=6),

Group B (n=7) was rats with i.p. ALA administration at 100 mg/kg,

Group C (n=7) was rats with topical ALA administration at 100 mg/kg.

A total of 7 rats were surgically induced to have the Barrett’s esophagus model (175-206 days post surgery) and were randomized into 2 groups:

Group D (n=3) for i.p. ALA administration at 100 mg/kg and,

Group E (n=4) for topical ALA administration at 100 mg/kg.

The topical administration procedure was illustrated in Figure 4.4. Rat anesthesia was induced and maintained by isoflurane (1.5% isoflurane with 98.5% O₂) throughout the entire procedure. The esophagus was isolated by polypropylene 7-0 sutures. ALA (0.2ml at 100mg/kg) was injected into the esophagus at the point illustrated in Figure 4.4 b and d. The rats were kept on warming-pad for 2 hours.
Figure 4.4 The surgical procedure demonstrated in this diagram was used to occlude the esophagus for administrating ALA in a topical manner. Topical administration of ALA in is illustrated. in (a) & (b) for the normal esophagus, and (c) & (d) showing the same procedure for the Barrett’s esophagus rats. The red line in (a) and (c) illustrates the suture position to isolate the esophagus. And blue arrows in all 4 figures illustrate the location where the ALA injection was done.

At 2 hours post ALA administration, the animal was humanely sacrificed and the esophagus, kidney and liver were extracted and used for ex vivo fluorescence scanner measurement (Typhoon™ 9410 by GE) for quantification of the production of PpIX in all relevant organs for the two methods of administration.
4.3.2 Results and Analysis

Fluorescence images and statistical assessment are listed in Figure 4.5 - 4.8. Based on the esophageal fluorescence images (Figure 4.5), normal rat esophagus with ALA administration (i.p. or topical), had higher PpIX fluorescence level than the control group esophagus as was expected. The fluorescence in Barrett’s esophagus (BE) was significantly higher, indicating a very high PpIX concentration generated on average, but spatial fluorescence heterogeneity was also observed in the Barrett’s esophagus. All of the Barrett’s esophagus tissues had higher PpIX fluorescence in the lower region of the esophagus (the upper section in images) as compared to the upper esophagus. In the Barrett’s groups, 2 animals showed distinctly less fluorescence. This heterogeneity could be induced not only by ALA initial topical injection position, but also by variation in the abnormal epithelium. During the experiment of topical ALA administration, reflux of the applied liquid was observed in all animals, which indicated that applied ALA was possible to contact whole esophagus.
Figure 4.5 Fluorescence images acquired by fluorescence scanner (Typhoon 9410, GE) 2hr post ALA administration with a dose of 100 mg/kg. Shown are (a) the control group (n=6); (b) normal rat esophagus with i.p. ALA administration (n=7); (c) normal rat esophagus with topical ALA administration (n=7); (d) rat Barrett’s esophagus with i.p. ALA administration (n=3); (e) rat Barrett’s esophagus with topical ALA administration (n=4).
Figure 4.6 The average PpIX fluorescence in esophagus at 2hrs post 100 mg/kg ALA administration. There was an increase of PpIX fluorescence with the ALA administration. Different administration methods did not show significant differences in either normal esophagus or induced Barrett’s esophagus. (p-value are listed in the text above, and animal used in group control: n=6; i.p. in normal: n=7; topical in normal: n=7; i.p. in BE: n=3; topical in BE n=4).

Statistical analysis was illustrated in Figure 4.6. Compared to the control group, there was a significant PpIX fluorescence increase at the 2 hour time point in both normal esophagus groups (p-value = 7×10^{-8} and p-value = 0.004) with the two different ALA administration methods. The Barrett’s esophagus groups showed higher PpIX fluorescence (p-value=8.6×10^{-10} and p-value=0.0001) as compared to the normal esophagus groups. The difference of the two ALA administration methods in the normal esophagus groups was not significant (p-value=0.33). Also no significant difference was found in Barrett’s esophagus groups with different ALA administration methods (p-value=0.47). Comparing the PpIX fluorescence heterogeneity between lower esophagus and upper esophagus, no significant difference was observed in the control group (p-
value=0.15), normal esophagus with i.p. ALA group (p-value=0.09), normal esophagus with topical ALA group (p-value=0.17) nor the group with Barrett’s esophagus using i.p. ALA (p-value=0.09). Interestingly though, the lower and upper esophagus had a significant difference in heterogeneity in the Barrett’s esophagus group with topical ALA administration (p-value=0.03).

Figure 4.7 Fluorescence images as acquired by the fluorescence scanner (Typhoon 9410, GE) 2hr post ALA administration with a dose of 100 mg/kg. In each image, the upper row is kidney sections and lower row is liver sections. In (a) is the control group (n=6); (b) the group with normal esophagus and i.p. ALA administration (n=7); (c) the group with normal esophagus and topical ALA administration (n=7); (d) the group with induced Barrett’s esophagus and i.p. ALA administration (n=3); and (e) the group with induced Barrett’s esophagus and topical ALA administration (n=4).
From the fluorescence image views, rats with ALA administration showed higher PpIX fluorescence in kidney and liver than the control group rats without ALA administration. It is interesting that in the non-Barrett’s group (normal esophagus), i.p. ALA administration yielded higher PpIX fluorescence in both kidney and liver, as compared to rats in the group with topical ALA administration. This difference was difficult to observe in groups with induced Barrett’s esophagus, due to saturation of the fluorescence. In the Barrett’s esophagus groups, high PpIX fluorescence was observed.

Figure 4.8 PpIX fluorescence in the kidney and liver 2hrs post 100 mg/kg ALA administration. There was an increase of PpIX fluorescence in the liver with i.p. ALA administration as well as in the Barrett’s esophagus group. Different administration methods did show significant difference in liver for normal esophagus groups (p-value=0.013). But no significant difference was observed in the liver within the Barrett’s esophagus groups (p-value = 0.50 and animal used in group control: n=6; i.p. in normal: n=7; topical in normal: n=7; i.p. in BE: n=3; topical in BE n=4).
The quantitative analysis of these images is illustrated in Figure 4.8, where in the normal esophagus tissue, the group with i.p. ALA administration showed higher fluorescence in liver as compared to the control group (p-value=0.017), however the group having topical ALA administration showed similar fluorescence in liver to the control group (fluorescence intensity 75 ± 6 and 74 ± 12 respectively). In groups with normal esophagus, i.p. ALA administration yielded higher PpIX fluorescence than the topical ones. In groups with Barrett’s esophagus, the average value of PpIX fluorescence in liver with i.p. ALA administration (227 ± 42) was higher than the topical ones (197 ± 60), with large standard deviation.

4.4 Discussion

*PpIX kinetics*

ALA-PpIX has a complex kinetic pattern which requires its own dosimetry pattern in photodynamic therapy. The PpIX kinetics in tissues[49-52, 87] have been studied for years, and yet for application to Barrett’s esophagus there is still need to better understand the dynamics which occur. A PpIX uptake peak in esophagus tissue was observed in the early hours after ALA administration, i.e. 2 hours post. Both the *in vivo* dosimeter and the *ex vivo* fluorescence instrument yield similar PpIX kinetics information, although in vivo signals peaked at 2 hours whereas the *ex vivo* data showed a subtle peak at 4 hrs. However the general trend was certainly similar which helps to
verify the performance of the \textit{in vivo} dosimeter. High variation in our measurements also indicated that the in vivo situation is complex, likely from inter-animal variation in PpIX productivity. Real-time estimation \textit{in vivo} of the PpIX signal will be shown to be important in work in later chapters of this thesis.

In this study, the ALA-PpIX kinetics in plasma were also investigated. A high level of PpIX in the plasma was observed at 2 hours post ALA administration, and remained at the same level for the 24 hour period studied. This is different from reports by several investigators who generally see a decrease in PpIX over the 24 hour period. Lofgren \textit{et al.} showed the highest levels of ALA induced PpIX in the plasma of rabbits occurred 1 hour post ALA intravenous (i.v.) administration of 50 mg/kg or 100 mg/kg, and 2 hour after a dose of 200 mg/kg. The PpIX concentrations declined to the control level by 24 hours\cite{88}. Henderson \textit{et al} also reported a similar kinetic pattern. The value of serum PpIX over a 5-hour period after an i.p. dose of 1000 mg/kg ALA was similar to that just after an i.v. dose of 7 mg/kg exogenous PpIX\cite{89}. Webber \textit{et al} reported a pharmacokinetic study of ALA-PpIX in 4 cancer patients after oral administration of 60 mg/kg ALA. They found that the half-life of exogenous ALA induced PpIX was approximately 8 hours after a brief distribution phase\cite{90}.

The PpIX kinetics in plasma could also explain the high PpIX observation in kidney and liver of rats in the group of Barrett’s esophagus with topical ALA administration. Barrett’s esophagus tissue has high PpIX productivity, and so PpIX should be re-distributed through vascular system with a higher accumulation expected in
the liver and kidney. More photosensitizer toxicity studies should be carried out to better understand the dose limiting factors and potentially to minimize the use of systemic ALA distribution, thereby improving local PpIX accumulation while minimizing the amounts in dose limiting organs.

*Systemic administration vs. topical administration*

An earlier study has shown that the plasma concentration of ALA (26.8 umol/L) peaks at 60 minutes after a single oral administration of 3.3 mg/kg ALA, as studied in a normal human subject, with a half-life of 50 minutes[91]. It was reported that exogenous ALA may penetrate across the blood-brain barrier and the central nervous system itself may synthesize porphyrins from exogenous ALA[92-94]. Therefore, care should be taken in planning clinical trials of systemic ALA administration, particularly for the patients with porphyria or severe disease of the liver and kidneys, because acute attacks of hepatic porphyrias with neurovisceral symptoms are always associated with high urinary excretion of ALA[95].

Our study of PpIX uptake comparison between ALA i.p. and topical administration also showed higher PpIX fluorescence occurs in the liver and kidney when ALA was administered i.p.. Since that topical administration limits ALA to the esophagus epithelium initially, topical delivery should help to lower PpIX levels in the liver and kidneys. It is not clear that these are the dose limiting organs, yet they are certainly indicative of whole body exposure to PpIX.
In groups of rats with Barrett’s esophagus, high PpIX productivity was observed in the diseased epithelium, as compared to the normal esophagus. Also high PpIX concentration was measured in the liver and kidney, where it is possible that PpIX redistribution via the blood stream from esophagus could explain this.

Even though there was no PpIX concentration difference in the esophagus with i.p. or topical ALA administration, there were different PpIX concentrations observed in liver and kidney. Our study suggested that using topical photosensitizer administration in the treatment of Barrett’s esophagus might reduce the potential of systemic photosensitizer toxicity issues.

*PpIX heterogeneity in Barrett’s esophagus*

Significant PpIX heterogeneity was also observed in the induced Barrett’s esophagus model. Two possible factors could induce this heterogeneity including: (i) problems with delivery of ALA to the esophagus, and (ii) spatial variation in cellular PpIX productivity. During the experiment of topical ALA administration, reflux of the applied liquid was observed in all animals. Thus we assumed the applied ALA was in the entire esophagus during 2 hour experiment. However there is still some uncertainty about how homogeneously the drug was distributed during the procedure, and this doubt will always be present in any in vivo topical application where good visualization is not possible. However in humans the ability to visualize the process with endoscopy is reasonably good and may eliminate this doubt if used in humans.
Barrett’s esophagus tissue has an imbalance of porphobilinogen deaminase (PBG-D) activity with that of ferrochelatase (FC), therefore leading to greater levels of Protoporphyrin IX productivity[79]. To investigate spatial PpIX heterogeneity productivity, the esophagus sections were cut at certain positions, allowing H&E pathology sections to be coregistrated to the PpIX fluorescence, as illustrated in Figure 4.9.

Based on the microscopic assessment of this esophageal tissue, “diseased” epithelium was found in (b) (c) (d) and (e). (b) and (c) showed high degree “diseased” epithelium, which correlated to high PpIX fluorescence, while (d) and (e) showed an inverse case. At position f, the epithelium was considered as “normal” tissue and it was also correlated with less fluorescence intensity as 95 (with a scale of 256), which is very close to the fluorescence value (127 ± 11) in normal esophagus with same ALA administration method and dose. Thus it is believed that the PpIX concentration spatial heterogeneity was mainly caused by heterogeneity in the level of dysplasia present in the esophagus, and thus was a true reflection of the disease state. Figure 4.10 illustrated that there was a positive relation between PpIX fluorescence and esophagus columnar epithelium height. However further quantitative study of this is required to make solid conclusions.
Figure 4.9 (a) PpIX fluorescence macro images of Barrett’s esophagus after 100 mg/kg ALA administration, where the letters b-f mark the approximate positions where microscopic images were taken. In (b)-(f) the microscopic images with H&E stain, related to the marked area of b-f in (a).
Figure 4.10 PpIX fluorescence intensity has positive dependence on the esophagus columnar epithelium height. Total 7 animals with Barrett’s esophagus model was under investigation here.

In summary, ALA induced PpIX signals were investigated in situ. Our study suggested that at 2 hours post ALA administration provided an optimal time to reach maximum PpIX signal in rat tissue. Our in vivo dosimeter was useful for PpIX concentration assessment. PpIX variations are also observed within individual animals and between animals, which suggests that the application of an in vivo dosimeter might improve the dosimetry situation in this type of therapy. PpIX heterogeneity was highest in the induced Barrett’s esophagus, and this was thought to be indicative of the high
heterogeneity of that dysplastic tissue. Two ALA application methods were studied, and topical administration was considered as a better possible choice to prevent photosensitizer toxicity to subjects, although the logistics of robust topical delivery implementation are likely the key next challenge in this area.
Chapter V Protoporphyrin IX Photobleaching and Its Effects on Photodynamic Therapy in Normal Rat Esophagus

In the past two decades the incidence of esophageal adenocarcinoma has increased dramatically, whereas survival rates have remained poor. Esophagus resection is the treatment of choice for esophageal cancer[96, 97], but an experimental alternative to surgery for intraepithelial carcinomas or precursor lesions is use of 5-aminolevulinic acid (ALA) induced Protoporphyrin IX (PpIX) photodynamic therapy (ALA-PpIX PDT)[28, 74, 98, 99]. Before light treatment, 5-aminolevulinic acid (ALA) is administered, which induces accumulation of the endogenous photosensitizer Protoporphyrin IX (PpIX). Subsequently, the esophagus is illuminated with light of a suitable wavelength (635 nm) at a defined fluence and fluence rate. Activated oxygen species, generated by ALA-PpIX PDT, notably singlet oxygen, act on critical cellular components, resulting in epithelial ablation. The result of ALA-PpIX PDT for Barrett’s esophagus was still not ideal. Re-epithelization with normal squamous epithelium is observed in 68-89% of patients, with a median reduction in area of only 30%[100]. Therefore treatment of Barrett’s esophagus by ALA-PpIX PDT still needs to be improved if it is to become a routine clinical treatment modality.
The efficacy of ALA-PpIX PDT is determined by a variety of parameters, such as, the distribution and concentration of the photosensitizer at the time of treatment, the wavelength of light, the fluence and fluence rate, the availability of oxygen within the irradiation volume and the optical properties of the tissue at the treatment wavelength. Using standardized treatment parameters, results of ALA-PpIX PDT should be subject to minimal variation, but in our study on ALA-PpIX PDT for normal rat esophagus, large variations in outcome were observed. Both no response and completed ablation of the epithelium were found, using same photosensitizer and light parameters. Thus, investigating real-time PDT parameters in individual animals must become an important issue in PDT dosimetry.

Porphyridin photobleaching and photoproduct formation have long been subjects of active investigation in porphyrin photochemistry and in studies of photodynamic therapy[20, 53, 76]. Yet its effect on PDT is not very well interpreted quantitatively [55, 56]. The assumption is that photobleaching would reduce the PDT efficacy by reducing the PpIX concentration and hence the tissue singlet oxygen concentration during the PDT treatment. The effect of the photobleaching of PpIX to the PDT response is complicated, and the effect of fractionation of the PDT treatment is suspected to be useful but still not well validated in the esophagus.

In this chapter, we would investigate the mechanism of Protoporphyrin IX photobleaching, and its effects on ALA-PpIX PDT of normal rat esophagus. Using a real-time PpIX dosimeter, we can monitor the PpIX concentration during PDT treatment and
can investigate the relationship of efficacy relative to the laser fluence rate and PpIX photobleaching kinetics.

5.1 Mechanism of Protoporphyrin IX Photobleaching

Photobleaching could play an important role in modifying the effects of photodynamic oxygen consumption because irreversible destruction of the photosensitizer must reduce the rate of photon absorption. Moreover, as it is extremely difficult to monitor changes in oxygen, light and sensitizer simultaneously during therapy. These are implicit methods of defining and/or monitoring dosimetry during PDT by measurement of a quantity which depends on all or at least most, of the above factors. Photosensitizer photobleaching is one of the suggested implicit measurements that could allow determination of the biological response that a specific treatment protocol would evoke.

To employ photosensitizer photobleaching as a dose metric for PDT or to simply incorporate it into a dosimetry model, it is essential to understand how the different mechanisms that could be involved in this process affect the production and deposition of singlet oxygen.

As mentioned by many investigators, the mechanisms for the photobleaching involve oxidation of photosensitizer (PpIX)[9, 101, 102]. A simplified reaction scheme is presented below.
\[ S_0 + h\nu \rightarrow S_1 \]
\[ S_1 \rightarrow T_1 \]
\[ T_1 + ^3O_2 \rightarrow S_0 + ^1O_2 \]
\[ S_0 + ^1O_2 \rightarrow \text{photoproducts} \]

where \( S_0 \) is the photosensitizer ground state, \( S_1 \) is the photosensitizer excited singlet state, \( T_1 \) is the photosensitizer excited triplet state, \( h\nu \) is the excitation photon, and \( ^1O_2 \) is the singlet oxygen.

Briefly, assuming that \( ^1O_2 \) reacts only with the ground state of the photosensitizer, and noting that: a) in the absence of photobleaching, the photosensitizer concentration will be constant, and b) the loss of ground state absorption proceeds much slower than the rates associated with the primary photochemical reactions occurring during treatment, we could derive a simple photosensitizer population decay expression,

\[
\frac{d[S_0]}{dt} = -k_{os}[S_0][^1O_2] \tag{Eq. 5.2}
\]

where \( k_{os} \) is the bimolecular rate constant for \( ^1O_2 \) reaction with ground state photosensitizer.

Eq. 5.2 could be derived in the term of light fluence (dose) \( D \) (irradiation fluence rate \( \times \) irradiation time, J/cm):

\[
\frac{d[S_0]}{dD} = -k_{os}[S_0][^1O_2] \tag{Eq. 5.3}
\]

which yields

\[
[S_1](D) = [S_1](D = 0) \exp(-k_{as}[^1O_2]D). \tag{Eq. 5.4}
\]

Thus, the photosensitizer photobleaching, as a monoexponential decay, has a decay
constant (photobleaching rate/coefficient) $k$, which is a function of local $^1O_2$ concentration.

We note that since the $^1O_2$ concentration is dependent on the photosensitizer concentration, Eq. 5.2 represents a photobleaching mechanism which cannot be characterized by a constant photobleaching coefficient. Under PDT treatment conditions in which photodynamic oxygen consumption is significant enough to limit the above photobleaching (Eq. 5.2), a predominantly $^1O_2$-mediated photobleaching mechanism would be expected to become relatively inefficient. Unless other oxygen-independent bleaching mechanisms were to become important under conditions of low tissue oxygen tension in vivo, an irradiance-dependence to the rate of photobleaching would be anticipated such that the photodegradation of photosensitizer for a given fluence would be greater at relatively lower irradiance. At present, reports of experiments that have attempted to test this prediction for the case of ALA-PpIX in vivo are conflicted. Robinson et al described an irradiance dependency on the rate of PpIX photobleaching in normal mice skin and in UVB-induced skin tumors that is qualitatively consistent with an oxygen-dependent bleaching mechanism[20]. Sorensen et al and Iinuma et al found no evidence for an irradiance dependence of the efficiency of PpIX photobleaching over a wide range of irradiance[78, 103]. Other groups have previously observed a two-phase decay in photosensitizer photobleaching[104, 105]. There is no evidence that PpIX undergoes significant redistribution on illumination in vivo or that PpIX undergoes complex photobleaching kinetic. For vascular targeting PDT, it is known that through
vascular shutdown, changes occur within minutes in the blood supply of the irradiated tissue, thus creating differences in local oxygen concentration. However, if for any reason a change occurs in $O_2$ concentration, the photobleaching rate could change during ALA-PpIX PDT.

This complex PpIX photobleaching mechanism is important not only from a mechanism perspective but also for the ongoing evaluation of the potential of photosensitizer photobleaching to report useful PDT dosimetry.

5.2 Materials and Methods

Animal

The Dartmouth College Animal Care and Use Committee (IACUC) approved this animal study. Six-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and housed 3 per cage under standard laboratory conditions (room temperature, $22 \pm 2$ °C; relative humidity, $55\% \pm 5\%$ and 12-hour light/dark cycle). The rats were allowed to acclimate for 2 weeks before surgical intervention. Rat chow was withheld 24 hours before surgical intervention, and liquids were stopped on the morning of PDT treatment.

Photosensitizer

5-aminolevulinic acid (ALA) was the drug used in all studies. It was purchased
from Sigma Inc. in powder form and a stock solution was prepared at 60 mg/ml in sterile PBS. The stock solution of ALA was kept in the dark at 4°C and used throughout this study.

Experiment setup

A total of 60 normal Sprague-Dawley rats were used in this study. Animals were randomized into 9 groups and ALA was administrated at different doses (i.p. at 0, 50, 100 mg/kg). After 2 hour incubation time, normal rat esophagus was irradiated by 633 nm laser. The irradiation was delivered into lower esophagus through a 1 cm long diffused end tip fiber. A total of 20 J/cm light fluence was delivered to each rat with different fluence rates (25 mW/cm or 50 mW/cm) and different laser irradiation methods (continuous irradiation or fractionated irradiation with 1 min laser on/off interval). During the treatment, rats were anaesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). A passive fluorescence dosimeter was used during the PDT treatment; both local laser fluence rate and Protoporphyrin IX fluorescence were monitored. All rats were sacrificed at 48 hours after PDT and esophagus of each was extracted for histologic analysis.

Three control groups were studied:

**Group A** (n=6) without any ALA administration and light irradiation;

**Group B** (n=6) with 100 mg/kg ALA administration dose and no light irradiation;

**Group C** (n=6) with 0 mg/kg ALA administration dose and 20 J/cm light
irradiation (fluence rate at 50 mW/cm).

Six PDT treatment groups were studied:

**Group D** (n=7) with 100 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 50 mW/cm (continuous treatment);

**Group E** (n=7) with 100 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 25 mW/cm (continuous treatment);

**Group F** (n=7) with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 50 mW/cm (continuous treatment);

**Group G** (n=7) with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 25 mW/cm (continuous treatment);

**Group H** (n=7) with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 50 mW/cm (fractionated treatment);

**Group I** (n=7) with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 25 mW/cm (fractionated treatment).

**Passive dosimeter application**

The esophagus dosimeter (described in Chapter III) was used in the esophagus beside the treatment fiber during the PDT light delivery. As mentioned in the previous chapter, this dosimeter could monitor both irradiation light fluence rate and Protoporphyrin IX fluorescence. The system was illustrated in Figure 3.2, however only one detection sensor was used in the rat esophagus study (Figure 3.4 d). After rats were
anaesthetized, the both the PDT irradiation fiber and dosimeter detection fiber were
inserted down to rat lower esophagus together. During PDT treatment, measurements
were taken every 10 sec. The treatment laser fluence rate was measured through a 620 –
650 nm band pass filter, and the Protoporphyrin IX fluorescence was measured through a
690 nm longer pass filter. PMT detection was used in both channels for simultaneous
acquisition.

Quantifying of PDT damage

Rats were sacrificed by cardiac injection of 0.1 ml saturated KCl. The esophagus
was extracted and fixed in 10% formalin. The specimens were embedded in paraffin wax,
sectioned and stained with hematoxylin and eosin (H&E). Mucosa/submucosa edema was
considered as early PDT response. The area of edema was measured by software ImageJ.

Protoporphyrin IX photobleaching fitting

Photobleaching decay constants were measured by following the fluorescence
decay of the PpIX in the tissue during irradiation. The photobleaching data of ALA
induced PpIX was fitted with the following equation[103]:

\[ I(D) = I_0 e^{-kD} + I_{ph} \]

Eq. 5.5

where I(D) is the fluorescence signal after a given light dose D (spatial irradiance ×
irradiation time, J/cm²), I₀ is the fluorescence signal prior to irradiating (D=0), k is the
photobleaching decay constant, and I(ph) is a dose-independent background fluorescence
term which is attributed to the fluorescence of the photoproducts or other contributions.

As we mentioned in the previous section, PpIX photobleaching includes a two phase decay, with a rapid photobleaching phase at the early time and a slow decay phase at the later time. We used Eq. 5.5 to fit the early phase, which was hypothesized here to be an oxygen-dependent photobleaching phase.

5.3 Results and Analysis

5.3.1 ALA-PpIX-PDT induced damage

Based on a macroscopic assessment of the normal esophagus, 48 hour post ALA-PpIX PDT, no damage was observed in the control groups, but in treatment groups the esophagus wall was thicken and deflated (Figure 5.1). Mucosa/submucosa edema in the treatment groups was easily observed in both macroscopic and microscopic sections.
Figure 5.1 Macro views of normal esophagus 48 hours post ALA-PpIX PDT. In (a) the rat in control group A is shown, without any ALA administration and light irradiation. In (b) the rat in control group C is shown, without any ALA administration but with 20 J/cm light irradiation at 50 mW/cm. In (c) the rat in treatment group F is shown with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at a fluence rate of 50 mW/cm. In (d) the extracted individual esophagus from (a), (b) and (c) are shown from top to bottom. In the control groups no damage was observed, but in the treatment groups, the esophagus wall was inflamed.
Figure 5.2 The microscopic view of H&E stained cross-sections of normal esophagus, 48 hour post ALA-PpIX PDT. In (a) a rat in control group A is shown, without any ALA administration and light irradiation. In (b) and (c) cross sections of esophagus are shown from a rat in treatment group F with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 50 mW/cm. Since the PDT treatment laser fiber had a 1 cm diffusing tip end, about 1 cm of the lower esophagus was the irradiated area, shown in (c). While the upper esophagus received no light irradiation, so was considered as un-irradiated area, shown in (b). The treatment response/damage was significantly different between the irradiated area and un-irradiated areas in all individual animals. Compared to the control group, the esophagus in the un-irradiated area received almost no damage while a large area of edema appeared in the irradiated zone. In (d) showed a high magnification view (10X) of a rat esophagus in the group with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 25 mW/cm. Ablation of epithelium was observed in this case.

Since the PDT treatment laser fiber had a 1 cm diffused tip end, the 1 cm lower part of the esophagus was considered as irradiated area, while upper esophagus was
considered as un-irradiated area. Assessment of PDT damage was applied to these two areas separately. In control groups A B C (no light no ALA, ALA only and light only respectively), no edema in whole esophagus was observed. Throughout all animals, there was no edema observed in the un-irradiated area (upper esophagus), this was quantitatively significant (p-value < 0.05) as compared to the control group. In treatment groups D-I, a wide range of damage was observed in the irradiated area (lower esophagus), varying from no damage, edema of esophagus mucosa/submucosa to erosion of the esophageal surface (illustrated in Figure 5.2).

As illustrated in Figure 5.3, both mucosa/submucosa areas in lower and upper esophagus were measured. The lower esophagi with a large mucosa/submucosa area were compared to the upper esophagus and this indicated a significant edema response from the PDT in the irradiated zone was always observed. For treatment groups D to I, the lower esophagus edema areas were 0.66 ± 0.06 mm², 1.54 ± 0.91 mm², 0.70 ± 0.32 mm², 1.45 ± 0.26 mm², 1.63 ± 0.30 mm² and 1.32 ± 0.41 mm² respectively. For the lower fluence rate group, the edema area was larger compared to higher fluence rate group. And fractionated treatment group yield larger edema area compared to continuous treatment group. Large standard deviations were found, illustrating a wide range of damage even with same treatment pattern.
Figure 5.3 Cross sectional edema area of the esophagus is shown. The blue bars refer to mucosa/submucosa area in lower esophagus, where light was applied and subsequent enlargement was observed. The dark red bars refer to mucosa/submucosa areas in upper esophagus, where edema was rarely observed and the mucosa/submucosa area was at same value throughout all groups (including control group). For the lower fluence rate group, edema area was larger compared to higher fluence rate group. The fractionated treatment group yielded increased edema as compared to the continuous treatment group. (Animals used in group A: n=6; B: n=6; C: n=6; D: n=7; E: n=7; F: n=7; G: n=7; H: n=7; I: n=7).

5.3.2 Fluence rate measurements

The initial light fluence rates (25mW/cm and 50 mW/cm) were determined by dosimeter assessment in vivo. During the PDT treatment, fluence rate was measured every 10s in the control group C and the treatment groups D - I. As illustrated in Figure 5.4, the average fluence rate varied within individual animals and different groups. The average fluence rates measured in groups C - I were 54.2 ± 2.6 mW/cm, 53.5 ± 6.6 mW/cm, 26.9 ± 4.3 mW/cm, 51.1 ± 4.0 mW/cm, 27.4 ± 4.7 mW/cm, 50.7 ± 5.5 mW/cm and 27.0 ± 2.6 mW/cm. The highest variations were 16.2% and 17.0% in the 25 mW/cm groups (E and G). This may due to problems with the dosimeter sensitivity at lower light
irradiance levels, however. Other groups showed smaller variation less than 10%. Given the initial light fluence rate setup in vivo, the light fluence changed not significantly, and the light fluence rate itself could be considered almost a minor factor in PDT dosimetry, though the fluence rate affects other dosimetry issues like photobleaching.

Figure 5.4 In vivo PDT treatment light fluence rate changed during treatment with a maximum variation of 17%. The mean value of the fluence rate was similar to the initial in vivo light fluence rate set up (50 mW/cm and 25 mW/cm, respectively). (Animals used in group C: n=6; D: n=7; E: n=7; F: n=7; G: n=7; H: n=7; I: n=7).

5.3.3 PpIX photobleaching

Figure 5.5 shows the raw fluorescence kinetics of individual rats during 4 different PDT treatment groups. The PpIX fluorescence intensity was calculated into PpIX concentrations according to calibration curve (Chapter III), and then PpIX
concentration was normalized to its initial value right before PDT irradiation. The normalized PpIX concentration is plotted against the measured fluence of each individual animal. The kinetics of photobleaching during irradiation in the esophagus is quite complex. In continuous treatment groups, there appears to be an initial rapid phase of photobleaching (within the first 5 – 10 J/cm) in some animals followed by a second slower phase of photobleaching that persists for the rest of the irradiation. To allow the comparison of the kinetics between animals, monoexponential decay was fit to the rapid phase of photobleaching.

Figure 5.5 *In vivo* PpIX kinetics are shown during light delivery, where the dots are experiment data points and solid line is the monoexponential decay fitting to the early phase of PpIX photobleaching.
5.3.4 Effects of PpIX photobleaching in continuous PDT treatment

Figure 5.6 shows the results of individual PpIX photobleaching rates, from the first phase time decay, in the continuous light application treatment groups (D – G). The first phase photobleaching rates in both the 50 mW/cm groups (D and F) were $(2.64 \pm 1.36) \times 10^{-2} \text{ cm/J}$ and $(1.99 \pm 0.84) \times 10^{-2} \text{ cm/J}$. And in both 25 mW/cm groups (E and G), the fit photobleaching rates were $(0.91 \pm 0.35) \times 10^{-2} \text{ cm/J}$ and $(1.60 \pm 0.81) \times 10^{-2} \text{ cm/J}$. The mean photobleaching rates in the lower fluence rate groups (25 mW/cm) were less than that in the high fluence rate group (50 mW/cm), yet the 38% to 52% variation values indicate that there does not appear to be a significant difference in photobleaching based on fluence rate.

![Figure 5.6 PpIX photobleaching rates fitted to the first phase of PpIX decay. There is no significant fluence rate dependence for the first phase PpIX photobleaching, and the overall mean value is $0.029 \pm 0.013 \text{ cm/J}$. (Animals used in group D: n=7; E: n=7; F: n=7; G: n=7).](image)
5.3.5 Effects of PpIX photobleaching in fractionated PDT treatment

Figure 5.7 is a typical PpIX kinetic in fractionated PDT treatment with laser on/off every 1 minute. Instead of 2 phase decay kinetic, the PpIX kinetic in fractionated PDT groups keep repeating with a similar rapid decay phase in each PDT irradiation fraction. The implied assumption here is that the rapid PpIX decay phase is dependent on sufficient oxygen, while the latter phase is not. As shown in Figure 5.7, the photobleaching rates in each fraction are $\text{4.83}\times10^{-2}$, $\text{3.06}\times10^{-2}$, $\text{2.63}\times10^{-2}$, $\text{1.29}\times10^{-2}$, $\text{2.54}\times10^{-2}$, $\text{2.23}\times10^{-2}$ and $\text{2.24}\times10^{-2}\text{cm/J}$. 

Figure 5.7 PpIX kinetic in fractionated PDT. Repeating of rapid decay phase in each PDT irradiation fraction was observed. And photobleaching rates in each irradiation fraction were at similar value level.
Figure 5.8 Photobleaching rates in individual animals in the fractionated PDT group. The average photobleaching rate is higher for fractionated irradiation than for continuous irradiation, but there were still some irradiation fractions that had low photobleaching rates, resulted in large standard deviation bars. The fractionated PDT was done with 2 fluence rates 50 mW/cm (a) and 25 mW/cm (b).

Similar photobleaching rates were observed in each irradiation fraction, indicating
that the oxygen re-diffusion may be taking place during the fractionated “light-off”
periods. The high average photobleaching rates also indicate that high oxygen
concentrations are likely present during each of the individual irradiation fractions.

Assessments of individual animals in the fractionated PDT groups showed the
average photobleaching rates for each irradiation fraction was higher than those in
continuous PDT groups (0.039 ± 0.013 cm/J vs. 0.024 ± 0.003 cm/J, p-value=0.0008) .
However, there were still some irradiation fractions with slower PpIX decay, which
indicated insufficient local oxygen concentration.

The kinetics of PpIX in fractionated PDT groups is complex, with the oxygen re-
diffusion situation, the overall photobleaching rates are higher than continuous PDT.

5.3.6 PpIX Photobleaching with PDT Response

Considered that the photobleaching rate from the early rapid decay phase was
assumed caused by \(^1\text{O}_2\)-dependent photobleaching mechanisms, this photobleaching rate
should then be a function of the local \(^1\text{O}_2\) produced and could be a direct PDT dosimetric.
Illustrated in Figure 5.3 and 5.9, the fractionated PDT group had a higher photobleaching
rate (3.9±1.3 \(\times10^{-2}\) cm/J) and higher PDT response/edema area (1.51 ± 0.36 mm\(^2\)),
compared to the photobleaching rate (2.35±0.91 \(\times10^{-2}\) cm/J) and PDT response/edema
area (1.02 ± 0.58 mm\(^2\)) in the continuous PDT groups. The higher photobleaching rates in
the fractionated PDT groups indicate that there could be higher \(^1\text{O}_2\) concentrations with
the assumption of oxygen re-diffusion during the dark interval. Overall PDT response has some dependence with photobleaching rate, but confounding data occurs in some cases where small photobleaching rate and significant edema were also observed. The correlation between photobleaching rate and PDT response/edema area is weak with coefficient of 0.19 (p-value=0.319). The photobleaching kinetics in individual animals is different and complex, it should be using carefully as a PDT dosimetry factor.

Figure 5.9 Plot of PpIX photobleaching rate and PDT response (edema area). The blue stars refer to animals in continuous PDT groups, and green circles refer to animals in fractionated PDT groups. The red solid line is the regression line to the data, the correlation between PpIX photobleaching rate and PDT response is 0.19 (p-value=0.319, and animal number used in this model is 30).
5.4 Discussion of PpIX photobleaching and O$_2$ Consumption in PDT Treatment

Protoporphyrin IX photobleaching is an important PDT dosimetry factor, because the PpIX photobleaching involves with local photosensitizer, oxygen and light fluence rate, which are major parameters of dosimetry model. In this chapter, the focus was on evaluation of PpIX photobleaching kinetics during light irradiation and its affect upon the PDT response to investigate the mechanism underneath this complicated kinetic. Therefore, it would help us to improve our PDT dosimetry model and finally improve the ALA-PpIX PDT treatment for Barrett’s esophagus.

Two phase decay kinetics of PpIX photobleaching

In vivo photosensitizer photobleaching is complex and limited by many factors. In our studies, two phase decay kinetics of PpIX photobleaching was observed. There appears to be an initial rapid phase of photobleaching (within the first 5 – 10 J/cm) in some animals followed by a second slower phase of photobleaching that persists for the rest of the irradiation.

Considering photobleaching kinetics are a function of photosensitizer concentration, light fluence rate and oxygen concentration, the change or limitation of those variables would cause the changing of photobleaching kinetics. Our studies about PpIX uptake shows that all animals in PDT treatment groups have an initial PpIX uptake
of 2.9 ± 0.6 ug/ml (with 50 mg/kg ALA administration dose) and 2.8 ± 0.5 ug/ml (with 100 mg/kg ALA administration dose), with the background/endogenous PpIX of 1.63 ± 0.28 ug/ml. The initial PpIX uptake has 2 folds of the background, but the slower phase of PpIX decay only persists in PpIX above 70% of initial uptake. This indicates that sufficient photosensitizer exists during the entire photobleaching effect. Our studies on light fluence rates changing during PDT treatment (Figure 5.4) also illustrated a minor variation of light fluence rate, with less than 10% variation for most animals and a maximum variation of 17%. Thus the changing of local oxygen concentration becomes the major factor inducing the two phase decay of PpIX. With initial sufficient oxygen, singlet oxygen is produced and reacts with ground state photosensitizer, a rapid photosensitizer decay is formed at an early stage. With consumption of local oxygen, less oxygen re-diffusion and less local photosensitizer, the limitation of singlet oxygen production results in less reaction with ground state photosensitizer, which induces a slower decay of photosensitizer. This theory helps to explain our findings in fractionated PDT studies. The oxygen re-diffusion during each no-light interval results in similarly rapid PpIX photobleaching during each light irradiation fraction.

In the two phase decay of PpIX kinetics observed in our studies, the initially rapid PpIX photobleaching with relatively high photobleaching rate indicates oxygen-dependent PpIX photobleaching and large amount of singlet oxygen production, while the slower, even stable, PpIX decay kinetic at later stages of the PDT treatment indicates there may be low tissue oxygen concentration. And the change of photobleaching rate
indicates the oxygen concentration is altered in tissue at this change in phase.

*Oxygen dependent photobleaching for the rapid decay phase*

Given the assumption of sufficient local oxygen, the initial rapid PpIX decay was fit to an oxygen dependent photobleaching model with a monoexponential function, where the photobleaching rate is an indicator of singlet oxygen production. The singlet oxygen production is also correlated to the photons absorbed by the photosensitizer. Many studies have shown that photobleaching rate has some inverse correlation with fluence rate, the rate that photobleaching increases with decreasing fluence rate[106]. Although our studies did not show significant light fluence rate dependence, there are some other reports also showing no fluence rate dependence[78, 103]. These conflicting reports are likely reasonable due to the complexity of the *in vivo* situation, and how it will likely depend upon tissue oxygenation and hence the pathophysiology of the tissue. In ideal conditions, reducing the fluence rate reduces the photochemical demand for oxygen and allows more oxygen to diffuse into the irradiated volume during therapy. This principle has been used in studies on fractionated irradiation in ALA-PpIX PDT. Short dark intervals lead to an increase in the rate of photobleaching immediately after the dark interval, in which oxygen re-diffusion is thought to occur within the treatment volume. However, oxygen re-diffusion may be affected by many factors. The vascular shutdown due to PDT would result in limits to oxygen re-diffusion. If for any reason a change occurs in oxygen concentration, the photobleaching rate could change during ALA-PpIX
PDT. In some cases such as our fractionated PDT studies, comparable lower photobleaching rates occur after dark intervals, indicating possible low singlet oxygen production and limited oxygen re-diffusion during dark interval.

To gain more insight into this mechanism, oxygen measurements in the esophagus would be useful in future studies, although admittedly these would be challenging to implement and quite possibly impractical for clinical use.

*Photobleaching kinetics and PDT response*

As an indicator of singlet oxygen in the oxygen dependent photobleaching model, photobleaching rate was investigated and correlated to PDT response in our studies. Low availability of oxygen could explain the lower initial phase photobleaching rate and the absence of a PDT response in some animals, compared with high tissue oxygen availability and high initial phase photobleaching rate with high PDT response (Figure 5.9). Also in the fractionated PDT groups, the relatively high photobleaching rate was thought to be a result of oxygen re-diffusion and thus had a correlated high PDT effect.

A number of factors could have affected the availability of oxygen during treatment such as anesthesia. However, a small reduction of blood oxygen saturation will not affect local oxygen concentration significantly. The amount of oxygen in the PDT irradiation volume is more important than the oxygen saturation in blood. Another potential factor to change the availability of oxygen in esophagus is the application of the PDT treatment fiber and dosimeter detection fiber. Even though the total size of two
fibers is less than 3 mm, the rigid fiber end tips could still cause compression of the esophagus wall and decrease the blood and oxygen supply. This could explain some cases in our PDT treatments. With high initial PpIX uptake and stable light fluence rate, the rapid PpIX decay phase was not observed and also no PDT damage/response was observed from the esophagus histopathology sections. This may resulted from the low local oxygen caused by compression of the esophagus wall.

In summary, we have demonstrated the feasibility of monitoring real-time light fluence rate and PpIX concentration during the PDT irradiation in rat esophagus. The results show high variability of PDT response of rat normal esophagus, and that the PDT responses are not simply determined by the fluence and fluence rate. In the meanwhile, the PpIX photobleaching kinetics was studied. A two phase decay of the PpIX was observed and it was hypothesized that the photobleaching rate is an indicator of singlet oxygen in tissue and therefore could be used as a dosimetry metric. However, only a weak correlation between the recovered photobleaching rate and PDT response was demonstrated. The change of photobleaching rate between the early and later phase of the curve may indicate the transition in oxygen concentration in tissue during PDT light delivery. This transition provides the idea to redefine the dosimetry model according to the photobleaching rate changes, which is examined in detail in the next chapter.
Chapter VI  Real-time Photodynamic Therapy Dosimetry

6.1 Photodynamic Therapy Dosimetry

The photodynamic dosimetry models that have been developed here are based on the assumption that direct tissue cell death, mediated by $^{1}\text{O}_2$, is the primary determinant of the therapy outcome. There is a concept of a threshold dose of $^{1}\text{O}_2$ in the dosimetry model [26, 107]. This threshold dose refers to the minimum cytotoxic concentration of $^{1}\text{O}_2$, at which point cells receiving amounts of $^{1}\text{O}_2$ equal to or higher than the threshold dose will be damaged irreparably by the treatment, while those receiving $^{1}\text{O}_2$ less than the threshold dose will be able to survive treatment. Unfortunately, while the threshold of $^{1}\text{O}_2$ has been studied in vitro systematically, [26, 107], the ability to extend this to in vivo is limited by current tumors to measure these parameters. But assuming that the dominant concept is that the more $^{1}\text{O}_2$ available in treatment volume, the more photodynamic therapy response should be, this dosimetry model could be used here to estimate PDT response with real-time dosimetry parameter measurement.

The basic naïve assumption would be that the concentration of $^{1}\text{O}_2$ was proportional to the product of the administered photosensitizer concentration and the total delivered optical density or fluence. This simplistic dosimetry model was found to be valid only for a limited range of drug and light doses in the previous chapter[1]. This is
not surprising, since one of the basic assumptions for such a model is that photosensitizer and oxygen availability as well as light delivery remains constant throughout treatment, and the fluorescence evidence supports the facts that both oxygen and photosensitizer concentrations vary significantly during the light treatment.

Direct studies demonstrating the variability in time and/or space of the $^3\text{O}_2$ concentration, photosensitizer availability and light deliver to the treatment area during typical therapy protocols [1, 74, 76, 99] suggest that the dynamic character of these quantities should be incorporated in PDT dosimetry estimation. With the real-time light dosimeter tools [108-110] such as the photosensitizer fluorescence dosimeter[53, 76, 111], it is now possible to monitor the light fluence rate integrated with the active photosensitizer concentration. The passive esophagus PDT dosimeter was developed to monitor these fluence and photosensitizer kinetics during PDT treatment. In the dosimetry model, even without a direct method of $^1\text{O}_2$ availability measurement, our dosimetry model assumed the use of a direct measure of local photosensitizer concentration and local light fluence would improve the accuracy of photodynamic dose calculation. Inter-subject variations in these parameters resulted in different $^1\text{O}_2$ productivities in the treatment volume, which correlate to different photodynamic therapy response rates.

However, simple measurements of the light fluence rate and photosensitizer concentration may not always indicate $^1\text{O}_2$ production rates in the treatment volume. The $^1\text{O}_2$ production is related to the number of photons absorbed by the photosensitizer, but
also limited by tissue $^3\text{O}_2$ concentration. Without sufficient local $^3\text{O}_2$ concentration, the light fluence rate and photosensitizer concentration are not valid parameters to calculate PDT dose. More information is needed to apply into our dosimetry model to estimate validation of those real-time parameters.

As discussed in the previous chapter, photosensitizer photobleaching can play an important negative feedback role in modifying the effects of photodynamic oxygen consumption because irreversible destruction of the photosensitizer will inevitably reduce the photon absorption rate. More importantly, the photochemical destruction of photosensitizer also consumes local oxygen. It would be ideal to have $^1\text{O}_2$ as a direct parameter in PDT dosimetry estimation, since $^1\text{O}_2$ is the major cytotoxic factor relate to PDT response. While there have been very resent reports on $^1\text{O}_2$ measurement in vitro and in vivo [112], it is still not clear that measurement of this signal will be robust enough to be a routine dosimetry tool. Photosensitizer photobleaching kinetics contains information on $^1\text{O}_2$ productivities in the treatment volume. The photobleaching rate is an indicator of $^1\text{O}_2$ availability. Thus photosensitizer photobleaching could be an alternative, implicit method for determination of the biological response that a specific treatment protocol could induce, which is derived from a signal which is robust and easily measured.

Furthermore, the interdependence of light fluence, photosensitizer concentration and oxygen consumption contribute to a very complicated scenario for the definition of photodynamic dose. In this study, an in vivo fluorescence dosimeter was used to monitor
the integrated kinetics of photosensitizer and light fluence together, during 5-Aminolevulinic Acid - Protoporphyrin IX photodynamic therapy in rat esophagus model. The measured kinetics were quantitatively assessed for dosimetry estimation to improve the definition of the PDT dose, as a predictor of response to the treatment.

6.2 Materials and Methods

This dosimetry study was based on the data in Chapter V. Instead of investigating the photosensitizer photobleaching kinetics, the focus here is on investigating the correlation between PDT response and all dosimetry parameters, such as direct measurements of the fluence rate, photosensitizer concentration and photosensitizer photobleaching kinetics.

*Photosensitizer and light fluence rate monitoring*

The drug 5-aminolevulinic acid (ALA) was used in all studies, as purchased from Sigma Inc. in powder form, using a stock solution prepared at 60 mg/ml in sterile PBS. This stock solution was kept in the dark at 4°C and used throughout this study. ALA was administered i.p. into animals 2 hours pre PDT treatment.

The esophageal dosimeter (described in Chapter III) was used in the esophagus beside the treatment fiber. The system was illustrated in Figure 3.2, but only one detection sensor was used in the rat esophagus study (Figure 3.4 d). After rats were
anaesthetized, both the PDT irradiation fiber and dosimeter detection fiber were inserted down into the rat lower esophagus together. PpIX fluorescence was measured right before and during treatment. During PDT treatment, measurements were recorded every 10 sec. The treatment light fluence was also measured by the dosimeter.

Experiment setup

As detailed in Chapter V, 60 normal Sprague-Dawley rats were randomized into 9 groups with different ALA administration doses, light fluence rates and treatment methods. The treatment patterns were listed again as the following.

There were 3 control groups:

**Group A** (n=6) without any ALA administration and light irradiation;

**Group B** (n=6) with 100 mg/kg ALA administration dose and no light irradiation;

**Group C** (n=6) with 0 mg/kg ALA administration dose and 20 J/cm light irradiation (fluence rate at 50 mW/cm).

There were 6 PDT treatment groups:

**Group D** (n=7) with 100 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 50 mW/cm (continuous treatment);

**Group E** (n=7) with 100 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 25 mW/cm (continuous treatment);

**Group F** (n=7) with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 50 mW/cm (continuous treatment);
**Group G** (n=7) with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 25 mW/cm (continuous treatment);

**Group H** (n=7) with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 50 mW/cm (fractionated treatment);

**Group I** (n=7) with 50 mg/kg ALA administration dose and 20 J/cm light irradiation at the fluence rate of 25 mW/cm (fractionated treatment).

**Quantifying of PDT damage**

Rats were sacrificed by cardiac injection of 0.1 ml saturated KCl. The esophagus was extracted and fixed in 10% formalin. The specimens were embedded in paraffin wax, sectioned and stained with hematoxylin and eosin (H&E). Mucosa/submucosa edema was considered as an early surrogate indicator of PDT response. The area of edema was measured from digitized images of the cross sectional view of the esophagus, using the software ImageJ.

**Protoporphyrin IX photobleaching kinetics**

As mentioned in the previous section, PpIX photobleaching has a bi-phase decay, with clearly both rapid and slower decay components. Eq. 5.5 was used to fit the early phase, which was hypothesized here to be an oxygen-dependent photobleaching phase.

For each animal in the PDT treatment group, the photobleaching kinetics were recorded and fitted to monoexponential decay over the early part of the curve. These
kinetic parameters, such as the photobleaching rate and decay time were used to define the dosimetry model.

6.3 Results and Analysis

6.3.1 ALA-PpIX PDT response assessment

Both the lower part of the esophagus (irradiated area) and the upper part of the esophagus (un-irradiated area) were assessed for PDT induced damage. From the microscopic view of H&E stained histology images, no damage was observed in the upper part of the esophagus throughout all animals.

In treatment groups D-I, a wide range of damage was observed in the irradiated area (lower esophagus), varying from no damage, to edema of the esophagus mucosa/submucosa, to erosion of the esophageal surface (illustrated in Figure 6.1). In this treatment study, 12 out of the 42 rats in groups D-I did not show any damage in the esophagus, even when given the same nominal PDT treatment as the others.
Figure 6.1 The 10× microscopic view of the H&E stained cross-sections of normal esophagus, 48 hour post ALA-PpIX PDT. This set of images illustrates a wide range of PDT response. Compared to the control group rat (a), some rats with ALA administration and light irradiation show different PDT responses including no damage (b), edema in the mucosa/submucosa area (c) and epithelial cell ablation (white arrow) in (d).
Figure 6.2 The rat esophagus cross sectional edema assessment is shown. The blue bars refer to mucosa/submucosa area in lower esophagus, where light was applied and subsequent enlargement was observed. The dark red bars refer to mucosa/submucosa areas in upper esophagus, where edema was rarely observed and the mucosa/submucosa area was at same value throughout all groups (including control group). For the lower fluence rate group, edema area was larger compared to higher fluence rate group. The fractionated treatment group yielded larger edema area compared to the continuous treatment group. (Animals used in group A: n=6; B: n=6; C: n=6; D: n=7; E: n=7; F: n=7; G: n=7; H: n=7; I: n=7).

Figure 6.2 is repeated here for clarity, but this time the focus is on the variation of the PDT response in each group. As mentioned in previous chapter, the lower esophagus exhibited a large induction in mucosa/submucosa area as compared to the upper esophagus and this indicated a significant edema response from the PDT in the irradiated zone. For same treatment pattern in individual animals, it would be desirable to reach a similar PDT result in each. But from our study, large standard deviations were found, illustrating a wide range of damage even with same treatment pattern. For control groups A, B and C, the variations were 10.2%, 5.3% and 17.5% respectively. This is considered consistent because rats in the control groups did not receive any PDT treatment. The light only control group (group C) had high variation (17.5%) possibly due to the reaction of
endogenous PpIX reactions with ambient light or dark chemistry due to the production itself. In the PDT treatment groups D – I, the variations were 8.7%, 59.1%, 45.2%, 18.2%, 18.6% and 31.0% respectively. To investigate the reason for high variation in individual animals, additional parameters other than treatment pattern should be used in our dosimetry model to calculate PDT dose.

6.3.2 PpIX uptake in PDT dosimetry

Since different animals have individually variant photosensitizer uptake and bleaching kinetics, it is necessary to calculate PDT dose based on local photosensitizer concentration instead of administration dose. As an endogenous photosensitizer, several major factors affect the PpIX yield, including the ALA access to the tissue, the PpIX productivity of the particular tissue, the yield of heme. These all contribute to make PpIX yield highly variable between individual animals.

In this study, the PpIX fluorescence was measured in each animal before PDT irradiation. Figure 6.3 illustrates the PpIX signal in esophagus, with different ALA i.p. administration doses (50 mg/kg and 100 mg/kg), where the PpIX yield was higher (2.91 ± 0.62 mg/kg and 2.83 ± 0.49 mg/kg) than in the control group without ALA administration (1.63 ± 0.28 mg/kg). The standard deviations in all three groups are in the range of 17-21%, with 17% in the control group, 21% in animals with 50 mg/kg ALA administration and 17% in animals with 100 mg/kg ALA administration. This level of variation indicates
different PpIX productivities in individual animals and might help to explain the variation in PDT response.

For comparison, a simplified dosimetry model was initially assumed so that the concentration of $^1$O$_2$, as a primary PDT damage factor, is proportional to the product of the local photosensitizer concentration and the total delivered photon density or fluence. This simple dosimetry model is calculated as follows:

$$PDT\_Dose = C_{PpIX} \times fluence$$  \hspace{1cm} Eq. 6.1

Using the PpIX concentrations immediately prior to PDT treatment in this calculation, PDT dose for individual animals for PDT treatment was calculated with the same 20 J/cm$^2$ fluence delivered. As illustrated in Figure 6.4, in this simplified dosimetry
model, PDT dose did not show a linear correlation with PDT response (edema area). The correlation coefficient was 0.06, which is too small a value to announce the correlation. This result suggested further investigation in more parameters involved in PDT dosimetry.

![Figure 6.4 Illustration of the lack of correlation between PDT response (edema area) and the simple model for PDT dose is shown, which was calculated with the initial PpIX concentration in each animal. Each data point is an individual animal. The correlation coefficient is 0.06 (p-value=0.757 and animal number used in this model is 30). Further modification is needed to optimize PDT dose calculation.](image)

6.3.3 Using Real-time PpIX kinetics in PDT dosimetry

Considering both PpIX concentration and light fluence rate are dynamic parameters during PDT treatment, it is not accurate only applying initial PpIX concentration and total light fluence in the PDT dose calculation. Using a passive esophagus dosimeter, dynamic values for PpIX concentration and light fluence rates
become accessible. Figure 6.5 illustrates the type of data available, acquired every 10 seconds, for both kinetics of PpIX fluorescence and remitted light fluence rate in one rat. This rat received 50 mg/kg ALA i.p. administration and light irradiation at 25 mW/cm for 800 seconds. PpIX photobleaching was observed. The PpIX signal decreased 35% during treatment, while light fluence rate changed a bit within 4% range.

Based on the kinetics of PpIX and fluence rate during light irradiation period, a superior way to calculate the PDT dose was to integrate the two signals for each animal as follows:

$$PDT \_ Dose = \int_{t_0}^{t_1} C_{\text{PpIX}}(t) \cdot \text{fluence \_ rate}(t) dt$$  \hspace{1cm} \text{Eq. 6.2}

where $C_{\text{PpIX}}(t)$ is the time-dependent PpIX concentration and $\text{fluence \_ rate}(t)$ is the fluence rate at all times, t. This improved PDT dosimetry model was used to calculate the PDT dose as an integration of the PpIX concentration and light fluence rate over the whole treatment time.
Figure 6.5 The kinetics of PpIX and fluence rate during PDT treatment in one rat. The animal received 50 mg/kg ALA i.p. administration and light irradiation at 25 mW/cm for 800 seconds. The PpIX signal decreased very quickly from 3.4 ug/ml to 2.2 ug/ml during the treatment, while the light fluence rate changed very little overall, within 23.6 mW/cm to 24.4 mW/cm.
Figure 6.6 An illustration of the poor correlation between PDT response (edema area) and PDT dose, which is calculated by integrating the PpIX signal with the fluence rate in individual animals. Each data point is an individual animal. The correlation coefficient is 0.09 (p-value=0.881 and animal number used in this model is 30), which indicates a poor correlation. Further modification is needed to yield a PDT dose calculation that is predictive of response.

PDT response (edema area) was plotted as a function of PDT dose in Figure 6.6. A weak correlation coefficient of 0.09 (p-value=0.881) also did not indicate a strong correlation between this PDT dose calculation and PDT response. The integration function in this dosimetry model is reasonable, but the integration time period might be the next most important parameter to consider changing.

6.3.4 Analysis of the effective treatment time in PDT dosimetry

In our histology analysis of PDT response, 12 out of 42 rats in treatment groups
D-I did not show any damage in the esophagus, even with high PpIX signals present. Figure 6.7 a. illustrates the PpIX kinetics in this case: after light irradiation, a rapid decay phase was not observed in the PpIX kinetics. And no PDT response was observed as shown in Figure 6.7 b. The other rats without any PDT damage also show similar kinetics.

PDT response (edema) was observed (as illustrated in Figure 6.7 d) in the other 30 rats in the treatment groups. The PpIX kinetics during treatment were investigated. The two phase decay kinetics of PpIX photobleaching were observed (illustrated in Figure 6.7 c). There appeared to be an initial rapid phase of photobleaching (within the first 5 – 10 J/cm) in some animals followed by a second slower phase of photobleaching that persisted for the rest of the irradiation.

As discussed in Chapter V, the initially rapid PpIX photobleaching with relatively high photobleaching rate indicates oxygen-dependent PpIX photobleaching, and hence large amounts of singlet oxygen production, while the slower, even stable, PpIX decay kinetic at later stages of the treatment indicates there may be low tissue oxygen concentration. Since singlet oxygen is a direct factor mediating PDT damage, the rapid decay phase in PpIX photobleaching could be considered as an effective treatment period.
Figure 6.7. Two situations were observed in the PDT treatment groups, with 12 out of 42 rats not showing any PDT damage even when having high PpIX signals and light irradiation. In this situation, the PpIX kinetics (a) did not have a rapid decay phase after irradiation and histology analysis did not find any PDT damage (b). PDT response (edema) was observed in another 30 rats in the treatment groups (d). In these 30 rats, a rapid decay phase of PpIX kinetics were observed (c), with the red line in (c) defining an effective transition point ending the rapid PpIX decay phase. This rapid decay phase could be considered as an effective PDT treatment period, because this phase is also considered to be the $^1\text{O}_2$ dependent photobleaching time, as discussed in Chapter V.

Thus, the dose estimates were recalculated, still using Eq. 6.2 to integrate the PDT dose, but using the rapid decay phase time limit as the integration time interval, instead of
whole PDT treatment time. The PDT response was then plotted as a function of this “effective” PDT dose, and is shown in Figure 6.8. The correlation coefficient for this data is then 0.45 (p-value=0.029), indicating a positive linear relationship between PDT response (edema) and PDT dose in our dosimetry model.

![Figure 6.8](image)

Figure 6.8 The data is plotted to illustrate the correlation between PDT response (edema area) and the “effective” PDT dose, which is calculated by integrating the PpIX signal with the fluence rate in individual animals, but only for the duration of the rapid decay phase. The correlation coefficient is 0.45 (p-value=0.029 < 0.05 and animal number used in this model is 30), which indicates positive linear relationship between this modified PDT dose and response.

### 6.4 Discussion of Effective PDT Dosimetry

*Oxygen in PDT dosimetry*

That photodynamic therapy tissue damage mediated by $^1\text{O}_2$ is thought to be the primary determinant of the therapy outcome. There is a threshold dose of $^1\text{O}_2$ in the
dosimetry model. This threshold dose refers to the minimum cytotoxic concentration of $^1\text{O}_2$. Some studies have been done to investigate the lowest $^1\text{O}_2$ to induce PDT response. Niedre et al showed that approximate $5.8 \times 10^{10}$ molecules of $^1\text{O}_2$ per cell is the lowest $^1\text{O}_2$ concentration to induce ALA-PpIX PDT response in normal mouse skin[113]. Georgakoudi et al estimated approximately $7 \times 10^9$ molecules of $^1\text{O}_2$ per cell is the threshold in EMT6 spheroids treated with Photofrin-PDT[114], and Farrel et al estimated the threshold as $5 \times 10^8$ molecules of $^1\text{O}_2$ per cell in rat liver treated with Photofrin-PDT[107]. Another in vitro study estimated the threshold as $5.6 \times 10^7$ molecules of $^1\text{O}_2$ per cell to achieve $1/e$ cell death in OCI-AML5 cells with ALA-PpIX PDT[112]. The threshold of $^1\text{O}_2$ to induce cell death in ALA-PpIX PDT for esophagus has not been investigated, but in a clinical pilot study of porphyrin-PDT in human esophageal carcinoma, treatment under hyperbaric oxygen at a level of 2 absolute atmospheric pressures appears to have enhanced the efficiency of PDT[115].

There is little doubt that oxygen plays an important role in PDT dosimetry calculation. Yet, in our dosimetry model, it is difficult to give an absolute estimation of $^1\text{O}_2$ concentration in esophagus. The photobleaching kinetic, as an implicit indicator of tissue $^1\text{O}_2$ concentration availability, provided a possible alternative method to define the PDT dose threshold. The change of photobleaching rate between the early and later phase of the curve may indicate the transition in oxygen concentration in tissue during PDT light delivery.
**PpIX photobleaching kinetics in PDT dosimetry**

Discussed in the previous chapter, photobleaching rate, in the oxygen dependent photobleaching model, was investigated and correlated to PDT response in our studies. Low availability of oxygen could explain the lower initial phase photobleaching rate and the absence of a PDT response in some animals, compared with high tissue oxygen availability and high initial phase photobleaching rate with high PDT response.

![Photobleaching rate histograms](image)

Figure 6.9 Photobleaching rate histograms in two PDT response situations: PDT damage (edema) observed in 30 rats (a) and PDT damage NOT observed in 12 rats (b). In situation (a), the photobleaching rate was $0.029 \pm 0.013$ cm/J with a median of 0.027 cm/J. In situation (b), the photobleaching rate was $0.0042 \pm 0.0059$ cm/J with a median of 0.0018 cm/J.

PpIX kinetics in the treatment group (42 rats) were fit to the first 4 J/cm irradiation with a monoexponential decay. If the PpIX kinetic data failed in fitting due to high variation, the photobleaching rate was counted as effectively zero in the statistical analysis. Figure 6.9 illustrates the photobleaching distributions in 30 rats with PDT
response and 12 rats with NO PDT response. With PDT response group, the average photobleaching rate was 0.029 cm/J with a median of 0.027 cm/J, while in the animals without PDT response, the average photobleaching rate is 0.0042 ± 0.0059 cm/J with a median of 0.0018 cm/J. From those two histograms, 0.02 cm/J (0.023 cm/J is the lower confidence interval bound with the 99% confidence level in histogram (a)) could be set as a dosimetry threshold to determine minimum PDT response. To estimate more accurate dosimetry thresholds, more rat data is needed in future studies.

A number of factors could have affected the availability of oxygen during treatment such as anesthesia. However, a small reduction of blood oxygen saturation will not affect local oxygen concentration significantly. The amount of oxygen in the PDT irradiation volume is more important than the oxygen saturation in blood. Another potential factor to change the availability of oxygen in the esophagus was the application of the PDT treatment fiber and dosimetry detection fiber. Even though the total size of the two fibers is less than 3 mm, the rigid fiber end tips could still cause compression of the esophagus wall and decrease the blood and oxygen supply. This could explain rats without PDT response in our PDT treatments. With high initial PpIX uptake and stable light fluence rate, the rapid PpIX decay phase was not observed (Figure 6.7 a) and also no PDT damage/response was observed from the esophageal histopathology sections (Figure 6.7 b). This might have resulted from compression of the esophagus wall from the fiber placement, producing transiently ischemic tissue with low local oxygen.
**Effective PDT treatment time and Fractionated PDT**

In the two phase decay of PpIX kinetics observed in our studies, the initially rapid PpIX photobleaching with a relatively high photobleaching rate indicates oxygen-dependent PpIX photobleaching and large amount of singlet oxygen production, while the slower, even stable, PpIX decay kinetic at later stages of the PDT treatment indicates there may be low tissue oxygen concentration. The PDT reaction involved with singlet oxygen is not effective for whole PDT irradiation time. High singlet oxygen production indicates high toxic species in tissue relate to final PDT response. Thus photobleaching rate or rapid PpIX decay kinetic could be used as a dosimetry parameter to determine effective PDT irradiation time.

Fractionated PDT has been studied for years, many reports showed fractionated treatment could improve the PDT treatment effects due to the re-oxygenation during the dark interval[103, 116-118]. In this approach, fractionated treatments result in longer effective irradiation times with higher tissue oxygen. But not all irradiation fractions are effective. Some low photobleaching rates in several irradiation fractions were also observed in our study, which indicated insufficient oxygen re-diffusion during the dark intervals occurred or local oxygen was limited due to some other reasons.

**Dosimetry controlled PDT**

Our final aim is to optimize ALA-PpIX PDT in Barrett’s esophagus.
Understanding the role of the effective irradiation time in our dosimetry model, a dosimetry controlled PDT approach could improve the PDT response by extending effective irradiation time. The fractionated PDT pattern should be designed according to real-time photosensitizer kinetics and calculation of effective time, instead of planning by initial pre-treatment numbers. Status of oxygen re-diffusion could also be inferred by our dosimetry model. To verify this dosimetry controlled PDT hypothesis, future studies should be applied with in vivo oxygen measurements[113].

In summary, the PDT response is a complicated function of photosensitizer concentration, light dose and tissue oxygen. PDT treatment planning should not be simply designed according to the parameters such as the light dose, photosensitizer injection dose and the time between injection and light treatment. Our dosimetry model demonstrated that a PDT dose calculation should be limited to the effective irradiation time. The PpIX photobleaching kinetics were used in our dosimetry model. Based on the results and using this PpIX real-time dosimetry system, it is hoped that optimization of individual treatments could become routine and be applied in ongoing clinical trials.
Chapter VII  Conclusion and Future Works

This chapter summarizes the work carried out in this thesis at each stage of its development, and provides suggestions for subsequent work are discussed. Section 7.1 reiterates the main achievements of the work in the following five aspects: (i) development of rat Barrett’s esophagus model; (ii) instrumentation and calibration of a passive esophagus dosimetry system; (iii) 5-aminolevulinic acid induced Protoporphyrin IX measurement; (iv) Protoporphyrin IX photobleaching and its effects on photodynamic therapy in normal rat esophagus; (v) refined photodynamic therapy dosimetry model for treatment in esophagus. In section 7.2, the direction for the future work discussed within the context of problems and difficulties encountered during these developments.

7.1 Thesis summary

With recent trends of applying 5-aminolevulinic acid (ALA) induced Protoporphyrin IX (PpIX) photodynamic therapy for treatment of Barrett’s esophagus, real-time dosimetry of the treatment will become important, to optimize treatment planning and improve treatment efficacy. The animal model was developed and parameters involved in PDT dosimetry model were investigated.

The rat Barrett’s esophagus model was developed by esophagojejunostomy
surgery. Our study suggests that both duodenal and gastric secretions can be used to induce the development of Barrett’s esophagus. With esophagojejunostomy surgery, both duodenal and gastric secretions were refluxed into the esophagus thru anastomosis. With carefully operation, rats done with esophagojejunostomy surgery could survive long enough (30 weeks) to induce Barrett’s esophagus. Epithelial changes were observed as early as 60 days. The changes were characterized as hyperplasia, ulceration, inflammation and dysplasia in microscopy. The columnar epithelium was easily observed in macroscopy. The cause of death in a large number of rats is still not clear, but believed to be related to the procedual complexity of the operation.

A passively monitoring in vivo fluorescence dosimeter was successfully developed to measure dosimetry parameters, such as photosensitizer concentration and light fluence rate, during the PDT treatment. The calibration studies in liquid phantoms showed the feasibility of applying the dosimeter into future PDT dosimetry studies, including light dose measurement in vivo with simultaneous PpIX measurement.

ALA induced PpIX signals were investigated in situ. Our study suggested that 2 hours post ALA administration provided an optimal time to reach maximum PpIX signal in the tissue in these rat models. Our in vivo dosimeter was useful for PpIX concentration assessment. PpIX variations were also observed within individual animals and between animals, which suggests that the application of an in vivo dosimeter might improve the dosimetry situation in this type of therapy. PpIX heterogeneity was highest in the induced Barrett’s esophagus, and this was thought to be indicative and correlate to heterogeneity
of the dysplastic tissue. Two ALA application methods were studied, and topical administration was considered as a better possible choice to prevent photosensitizer toxicity to subjects, although the logistics of robust topical delivery implementation are likely the next key challenge in this area.

During PDT irradiation in the rat esophagus, we have demonstrated the feasibility of monitoring real-time light fluence rate and PpIX concentration. The results show high variability of PDT response in rat normal esophagus, and that the PDT responses are not simply determined by the fluence and fluence rate. All the same time, the PpIX photobleaching kinetics were studied. A two phase decay of the PpIX was observed and it was hypothesized that the photobleaching rate is an indicator of singlet oxygen in tissue and therefore could be used as a dosimetry metric. However, only a weak correlation between the recovered photobleaching rate and PDT response was demonstrated. The change of photobleaching rate between the early and later phase of the curve may indicate the transition in oxygen concentration in tissue during PDT light delivery. This transition provides an idea of how to refine the dosimetry model based upon measured photobleaching rate changes.

Finally, a PDT dosimetry model was refined by comparing different calculations of PDT dose with photosensitizer concentration, light dose and tissue oxygen. Our correlation studies of PDT response and PDT dose demonstrated that PDT treatment planning should not be simply designed according to the parameters such as the light dose, photosensitizer injection dose and the time between injection and light treatment. A
refined dosimetry model was demonstrated that provides a PDT dose calculation that should be limited to the effective irradiation time. The PpIX photobleaching kinetics were used in our dosimetry model to determine the effective irradiation time. Based on the results and using this PpIX real-time dosimetry system, it is hoped that optimization of individual treatments could become routine and be applied in ongoing clinical trials.

7.2 Future work

7.2.1 Topical administration of ALA

Our study of PpIX generation compared between ALA i.p. and topical administration also showed higher PpIX fluorescence occurs in the liver and kidney when ALA was administered i.p.. Since topical administration limits ALA to the esophagus epithelium initially, topical delivery may help to lower PpIX levels in the liver and kidney. PpIX in liver and kidney has two sources: one is the endogenous PpIX produced by exogenous ALA; and the other is exogenous PpIX which is re-distributed from PpIX in Barrett’s esophagus. High ALA in liver and kidney could result in high PpIX, but high PpIX in esophagus could also result in accumulation of PpIX in other organs through the vascular system. To interpret ALA/PpIX distributions in the administration study, more data are needed including ALA concentration in organs and plasma at different time-points post administration, and PpIX concentration in plasma. Methods to isolate endogenous and exogenous PpIX in liver and kidney would also help to determine the
ALA distribution in different organs. Low local ALA concentration but high PpIX concentration could be the result of PpIX re-distribution. This knowledge could help us to explain the high PpIX signals in liver and kidney in Figure 4.7 d and e, in rats with Barrett’s esophagus with topical administration.

In our study, topical delivery of ALA was carried out by a surgical technique. This is not recommended for clinical use but useful in animal study. For clinical treatment, it should be possible to develop a noninvasive topical delivery method. A catheter with balloon is an option for this. Using an inflated catheter balloon to close the end of the lower esophagus, could keep ALA on the targeted epithelium. But effects of balloon pressure to nearby vascular and other organs should be carefully studied.

### 7.2.2 PpIX photobleaching and singlet oxygen *in situ*

In the two phase decay of PpIX kinetics observed in our studies, the initially rapid PpIX photobleaching with relatively high photobleaching rate indicates that oxygen-dependent PpIX photobleaching and large amount of singlet oxygen production might be occurring, while the slower, even stable, PpIX decay kinetic at later stages of the PDT treatment indicates there may be a low tissue oxygen concentration. And the change of photobleaching rate indicates that the oxygen concentration is altered in tissue at this change in phase.

Without the measurement of tissue singlet state oxygen or triplet state oxygen, our
photobleaching model assumed the significant changing of photobleaching rate was result of singlet oxygen change. This assumption is not verified in vivo, though the correlation between PpIX photobleaching rate and singlet oxygen concentration in vitro studies has been reported. In vitro situation, oxygen is assessable for measurement and the concentration could be controlled in designed range. But in vivo situation, singlet/triplet oxygen kinetic is complicate and affected by many factors. It is important to investigate such correlation between oxygen and PpIX photobleaching. Niedre et al recently reported a possible detection of singlet oxygen in biological systems by its luminescence at 1270 nm[113]. Physical Sciences Inc. had developed a singlet oxygen measurement instrument based on this theory[119]. Coupled with photosensitizer/light fluence rate dosimeter, it become possible to measure photosensitizer, light fluence rate and singlet oxygen simultaneously. With the data of singlet oxygen, our PpIX photobleaching model could become more accurate to interpret the two phase decay which occurs during the treatment time.

7.2.3 Dosimetry controlled PDT treatment

The final aim of our dosimetry study was to optimize ALA-PpIX PDT in Barrett’s esophagus. Understanding the role of the effective irradiation time in our dosimetry model, a dosimetry controlled PDT approach could improve the PDT response by extending effective irradiation time. Fractionated PDT patterns should be designed.
according to the real-time photosensitizer kinetics and a calculation of effective time, instead of focusing on planning with initial pre-treatment numbers. Pre-clinical study of this proposed dosimetry controlled PDT treatment is needed to verify our dosimetry model. Animals received the same PDT dose with dosimetry controlled treatment and would result in PDT response with a smaller inter-subject variation.

Determination of the effective irradiation time is another issue that is needed to be investigated in a dosimetry controlled PDT treatment. Though we have compared the photobleaching rate between rats with and without PDT response (Figure 6.9), more work is still needed to determine the PDT dose threshold to induce a superior treatment response. Meanwhile, application of the feasibility of singlet oxygen measurement would help to refine the effective irradiation time.
APPENDIX A  *in vivo* Dosimeter System

A. Data Acquisition Hardware.

A DAQCard™-6024E (National Instruments, TX) was used in the dosimeter system for analog data acquisition and system control. The DAQ-6024E was a PCMCIA card for notebook applications with two 12-bit analog outputs and 8 digital I/O lines. The DAQ card was connected to a CB-68LP (National Instruments, TX) connection board with the same port numbers. The 6024E pin out is illustrated in Figure 8.1. The connections to the dosimeter PMTs and mechanical 6-1 switcher control board are illustrated in Table 8.1.

<table>
<thead>
<tr>
<th>Hardware description</th>
<th>DAQCard connections Port-name (pin number)</th>
<th>Input</th>
<th>Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence channel PMT gain setup</td>
<td>AO 0 (pin 22)</td>
<td>0~1.0 vol.</td>
<td></td>
</tr>
<tr>
<td>Fluorescence channel PMT measurement</td>
<td>AI 1 (pin 33)</td>
<td></td>
<td>0~10.0 vol.</td>
</tr>
<tr>
<td>Reference channel PMT gain setup</td>
<td>AO 1 (pin 21)</td>
<td>0~1.0 vol.</td>
<td></td>
</tr>
<tr>
<td>Reference channel PMT measurement</td>
<td>AI 3 (pin 30)</td>
<td></td>
<td>0~10.0 vol.</td>
</tr>
<tr>
<td>Analog ground</td>
<td>AI GND (pin 67, 29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AO GND (pin 55, 54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-1 mechanical switcher control</td>
<td>PO 0 (pin 52)</td>
<td>0 or 5.0 vol. TTL.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PO 1 (pin 17)</td>
<td></td>
<td>0 vol. enable shutter and 5.0 vol. disable shutter</td>
</tr>
<tr>
<td></td>
<td>PO 2 (pin 49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PO 3 (pin 47)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PO 4 (pin 19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PO 5 (pin 51)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digital ground</td>
<td>D GND (pin 50)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 8.1. The *in vivo* dosimeter system hardware to the DAQCard connections are listed above.
Figure 8.1 DAQCard™-6024E pin out schematic used.

B. Data Acquisition Software

Labview (Labview 6.0, National Instruments, TX) software was developed to control the data acquisition of both treatment laser fluence rate and PpIX fluorescence...
intensity. A typical measurement is followed the flow chart in Figure 8.2.

Figure 8.2 A flow chart of dosimeter measurement is shown.
Before each measurement, several parameters are setup as following:

**Filename**: Measurement data was saved automatically into the assigned file;

**Measurement sessions L**: This parameter determined how many sessions of measurement would be carried out. Each session of measurement contains one measurement of each detection fiber. If 6 detection fibers are enabled for the measurement, one session included 6 individual measurements, and total measurement number will be \( L \times 6 \).

**Detection fiber total number N**: This parameter determined how many detection fibers were used in the experiment. We had a maximum number of 6 fibers.

The Labview software subroutines are listed in Table 8.2:

<table>
<thead>
<tr>
<th>VI Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus dosimeter v3 auto 6 channels.vi</td>
<td>Main user interface for measurement. Input: filename, experiment comments and measurement session number.</td>
</tr>
<tr>
<td>Generate 1 Point on 1 channel.vi</td>
<td>Set the gain on the PMT. Input: device number (=1), channel number (=0,1), value to generate (gain=0~1.0). Output: gain value (=input &lt;value to generate&gt;).</td>
</tr>
<tr>
<td>Write 1 Point to Digital Line.vi</td>
<td>Set the shutter status. Input: device number (=1), digital line (=0~5), value (True=on, False=off).</td>
</tr>
<tr>
<td>Acquire 1 Point from 1 channel.vi</td>
<td>Data acquisition from PMT. Input: device number (=1), channel number (fluorescence channel=1, reference channel=3). Output: sample value (=0~10 vol. from PMT channel)</td>
</tr>
<tr>
<td>Write File.vi</td>
<td>Save data to a file. Input: filename, value to saved.</td>
</tr>
</tbody>
</table>

Table 8.2 The in vivo dosimeter software subroutine list.
BIBLIOGRAPHY


34. Marcus SL, Sobel RS, Golub AL, Carroll RL, Lundahl S, Shulman DG: Photodynamic therapy (PDT) and photodagnosis (PD) using endogenous...


Barrett NR: Chronic peptic ulcer of the oesophagus and 'oesophagitis'. Br J Surg 1950; 38: 175-82.


75. Gold MH, Goldman MP: 5-aminolevulinic acid photodynamic therapy: where we have been and where we are going. Dermatologic Surgery 2004; 30(8): 1077-83; discussion 1083-4.


102. Foote CS: Mechanisms of photosensitized oxidation. There are several different types of photosensitized oxidation which may be important in biological systems. Science 1968; 162(857): 963-70.


107. Farrell TJ, Wilson BC, Patterson MS, Chow R: The dependence of photodynamic


