

# Molecular imaging of photodynamic therapy

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## ABSTRACT

Recent advances in light sources, detectors and other optical imaging technologies coupled with the development of novel optical contrast agents have enabled real-time, high resolution, *in vivo* monitoring of molecular targets. Non-invasive monitoring of molecular targets is particularly relevant to photodynamic therapy (PDT), including the delivery of photosensitizer in the treatment site and monitoring of molecular and physiological changes following treatment. Our lab has developed optical imaging technologies to investigate these various aspects of photodynamic therapy (PDT). We used a laser scanning confocal microscope to monitor the pharmacokinetics of various photosensitizers in *in vitro* as well as *ex vivo* samples, and developed an intravital fluorescence microscope to monitor photosensitizer delivery *in vivo* in small animals. A molecular specific contrast agent that targets the vascular endothelial growth factor (VEGF) was developed to monitor the changes in the protein expression following PDT. We were then able to study the physiological changes due to post-treatment VEGF upregulation by quantifying vascular permeability with *in vivo* imaging.

**Keywords:** photodynamic therapy, optical imaging, molecular imaging

## 1. INTRODUCTION

PDT is an emerging therapy that shows promise in the treatment of various types of cancers including prostate cancer. In mice PCa models, prostatectomy in combination with PDT lead to a significant decrease in distant metastasis compared to prostatectomy alone (1). Human clinical trials of PDT in PCa resulted in a decrease in prostate specific antigen (PSA), both as a primary treatment (2) and in combination with radiotherapy (3). Currently, PDT using Photofrin® is clinically approved for the treatment of esophageal cancer, non-small cell lung cancer and high-grade dysplasia in Barrett’s esophagus. A number of other photosensitizers (PS) including benzoporphyrin derivative monoacid (BPD) are in human clinical trials.

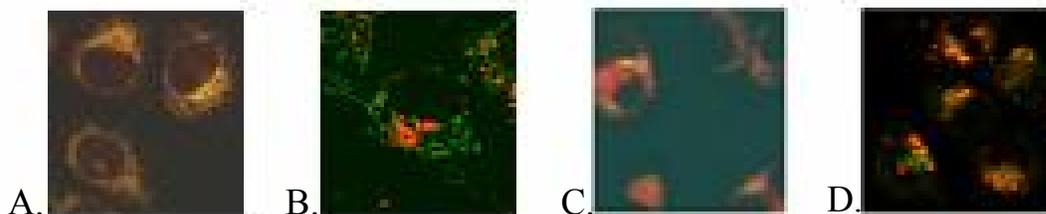
PDT is based on the concept of PS accumulation in the treatment site and photoactivation of the PS to generate active cytotoxic molecular species. After PS administration and preferential accumulation within the treatment site, irradiation of light at the appropriate wavelength excites the PS from the ground singlet state to the first excited singlet state. As the activated PS relaxes to the ground state, the energy is transferred to oxygen ( $^3\text{O}_2$ ) through collisional quenching and reactive oxygen ( $^1\text{O}_2$ ) toxic to cells and tissues is generated. The resulting reactive oxygen can destroy tumors.

## 2. IMAGING PHOTOSENSITIZER LOCALIZATION

PDT response depends on both the delivered light dose as well as the PS dose accumulated in the treatment site. Thus monitoring the accumulation of the PS in the treatment site can yield important insights into the subsequent treatment response. PS localization can be monitored using various optical methods that is capable of detecting the fluorescence emission of PS.

### 2.1 Imaging subcellular PS localization in *in vitro*

Our lab is working on a novel treatment modality by conjugating a therapeutic antibody with a PS to synergistically combine the effects of immunotherapy with PDT. However, the subcellular localization of the antibody-PS conjugates is not well understood. Recent studies have shown that localization of the PS in different subcellular compartments can lead to the induction of different pathways for apoptosis (4, 5). In order to better understand the subcellular localization of the antibody-PS conjugates, we monitored the accumulation of the conjugates in *in vitro* samples and compared them with the staining patterns of organelle specific markers.

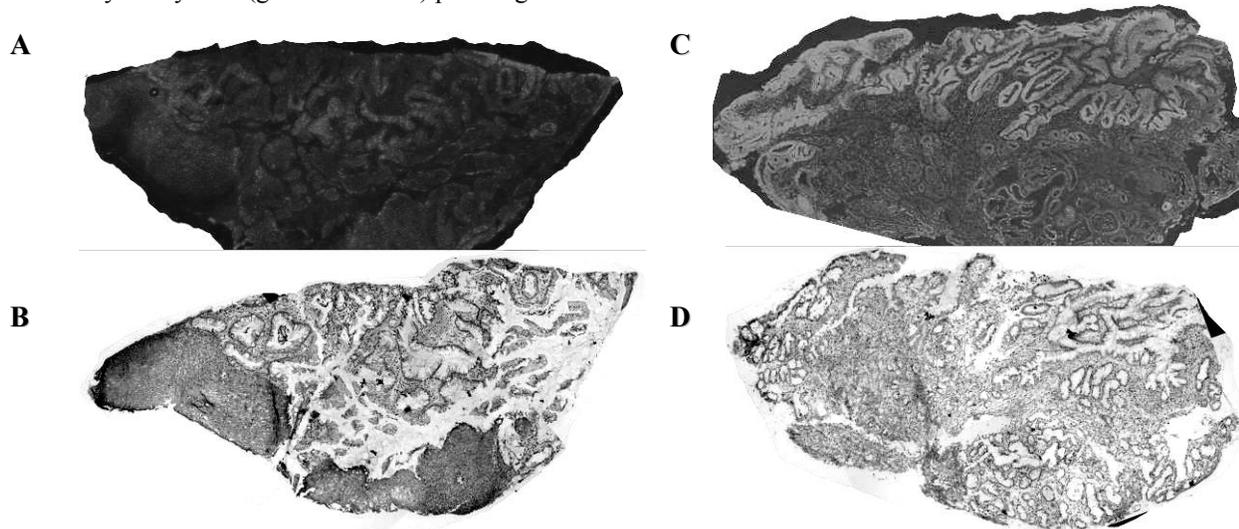


**Figure 1.** OVCAR-5 cells were incubated for 15h with 140nM equivalent of BPD either in its unconjugated (A & C) or conjugated form (B & D). The cells were also co-stained with organelle specific fluorescence markers for mitochondria (A & B) and lysosomes (C & D). Fluorescence from the markers is shown in false color as green and fluorescence from BPD is shown in false color as red. Regions of yellow indicate co-localization of the BPD fluorescence (shown in false color as red) and fluorescence from organelle markers (shown in false color as green) within the same subcellular site.

In Figure 2, OVCAR-5 ovarian cancer cells were incubated with either benzoporphyrin derivative (BPD) or BPD conjugated with C225, an anti-EGFR antibody. The mitochondria and the lysosomes in these cells were simultaneously labeled with organelle-specific markers. Fluorescence from the BPD and the organelle-specific markers were imaged using a confocal fluorescence microscope. After 15 hours of incubation, BPD by itself, shown in red, will co-localize with the mitochondria, which is labeled in green. However, BPD conjugated with C225 will localize in the lysosome, which is also labeled with an organelle-specific marker. Confocal laser scanning fluorescence microscope can be used as a valuable tool to monitor the subcellular localization of the PS with high spatial resolution.

## 2.2 Imaging PS localization in *ex vivo* tissue

Photodynamic therapy (PDT) using 5-aminolevulinic acid (ALA) is an emerging and a promising treatment modality for dysplastic Barrett's esophagus (BE). Administered orally, ALA, is converted into the photosensitizer, protoporphyrin IX (PPIX) *in situ* via the heme biosynthetic pathway. When activated with red light (635nm) PPIX creates oxygen radicals that cause cell death. Differentiation-dependent expression of some heme enzymes can enhance generation of PPIX from exogenously administered ALA, thereby increasing the therapeutic efficacy of ALA-PDT (6). Pre-treatment with differentiating agents, such as Accutane, can promote PPIX production by redirecting cells to their normal phenotypic maturation and may improve tissue destruction. PPIX fluorescence in tissue biopsies from the BE segment of patients were analyzed *ex vivo* using confocal laser scanning microscopy and the corresponding H&E staining of the sections were analyzed by a GI (gastro-intestine) pathologist.

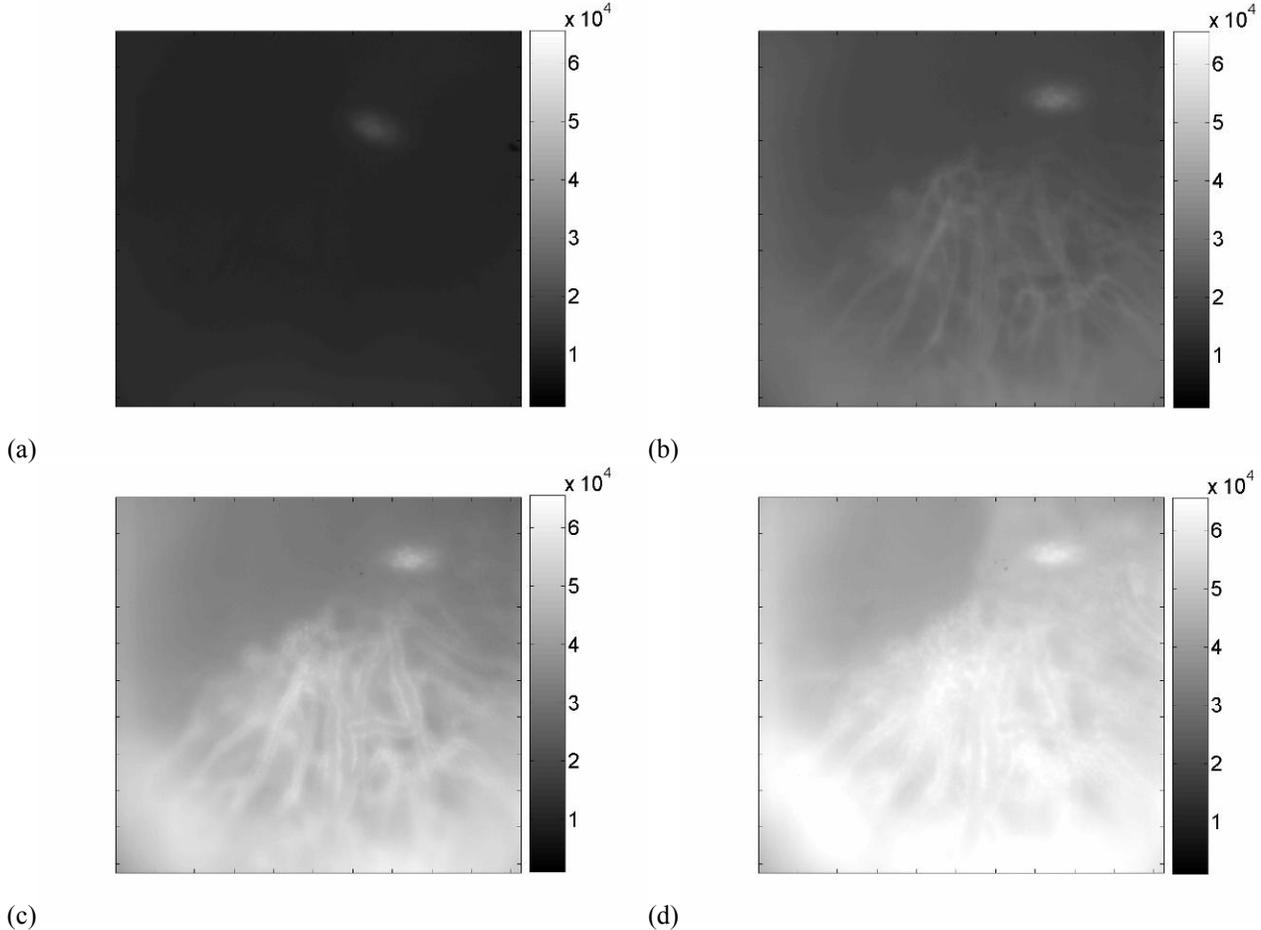


**Figure 2.** Comparison of PPIX Fluorescence between Patients as imaged on Confocal Laser Scanning Microscopy. (A) PPIX fluorescence in section of biopsy from Patient 3 who was not treated with Accutane. (B) H&E staining of A. (C) PPIX fluorescence in section of biopsy from Patient 6 who was pre-treated with Accutane. (D) H&E staining of C.

The results of the study showed increased PPIX fluorescence in BE tissue biopsies of patients pre-treated with Accutane, suggesting that differentiating agents can be used to increase the efficiency of PPIX production from ALA in BE. Additionally, we observed PPIX fluorescence was concentrated in the columnar epithelium of the *ex vivo* samples, suggesting that the PPIX production is high in the columnar epithelium.

### 2.3 Imaging PS localization *in vivo*

PDT can destroy malignant cells in the treatment site through different mechanisms depending on PS localization within the tissue. At late time points after PS injection, PS diffuses into the tissue and PDT results in direct tumor cell destruction (7) with limited vascular destruction (8). However at earlier time points after PS injection, PS is localized mostly in the vasculature, resulting in vascular shutdown (9) that leads to hypoxia (10) in the treatment site. By detecting the PS fluorescence using *in vivo* imaging tools, PS localization in the treatment site can be monitored *in vivo*.



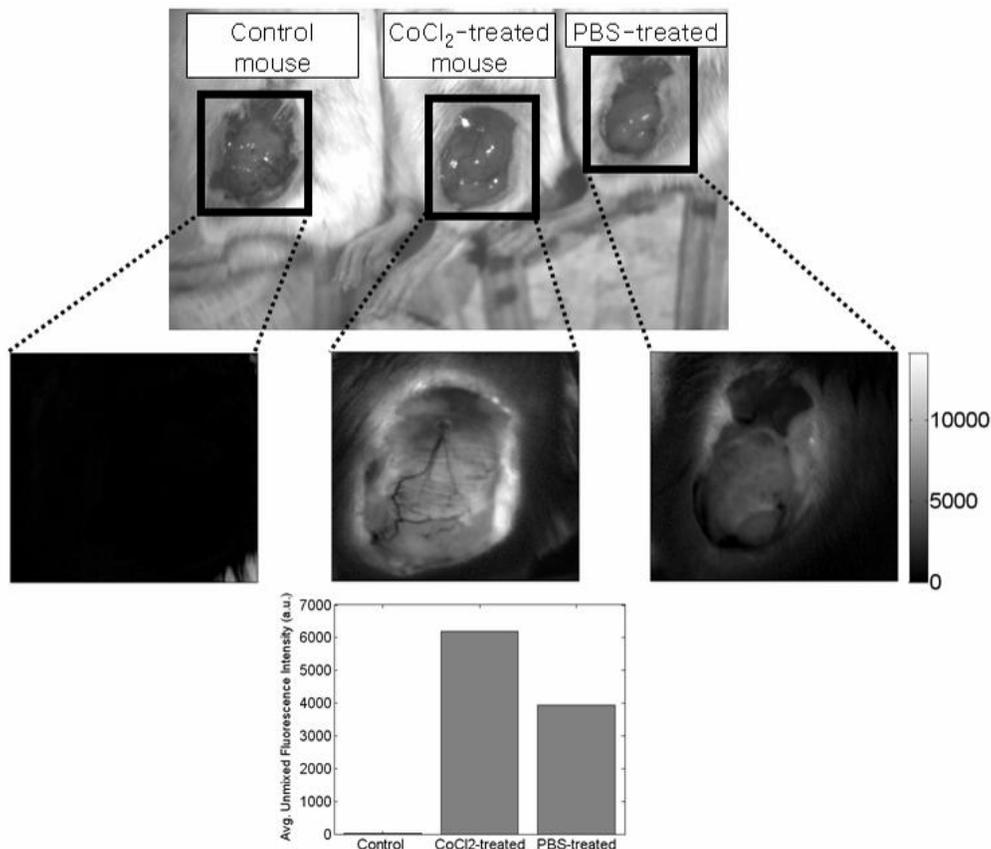
**Figure 3.** Images of delivery of ETNBs in collagen pellets following intravenous injection through the tail vein. Images were acquired (a) before ETNBs injection, (b) immediately after ETNBs injection, (c) 14 minutes after ETNBs injection and (d) 60 minutes after ETNBs injection from the identical imaging field.

In Figure 3, we developed a new *in vivo* model for granuloma by implanting collagen pellets infected with microbials subcutaneously in mice. In order to identify the characteristics of PS localization in the new *in vivo* model, the PS delivery in the collagen pellet was monitored using a custom-built intravital fluorescence microscope. The microscope is based on a long working distance objective that is suitable for *in vivo* imaging of small animals. After intravenous injection of the PS, the collagen pellet was imaged at various time points. At early time points following the intravenous injection, we observed that the PS is localized mostly in the vasculature. However, at later time points, much of the PS fluorescence was observed in the tissue surrounding the vasculature, suggesting that the PS has diffused out of the vasculature.

### 3. IMAGING MOLECULAR EFFECTS FOLLOWING PDT

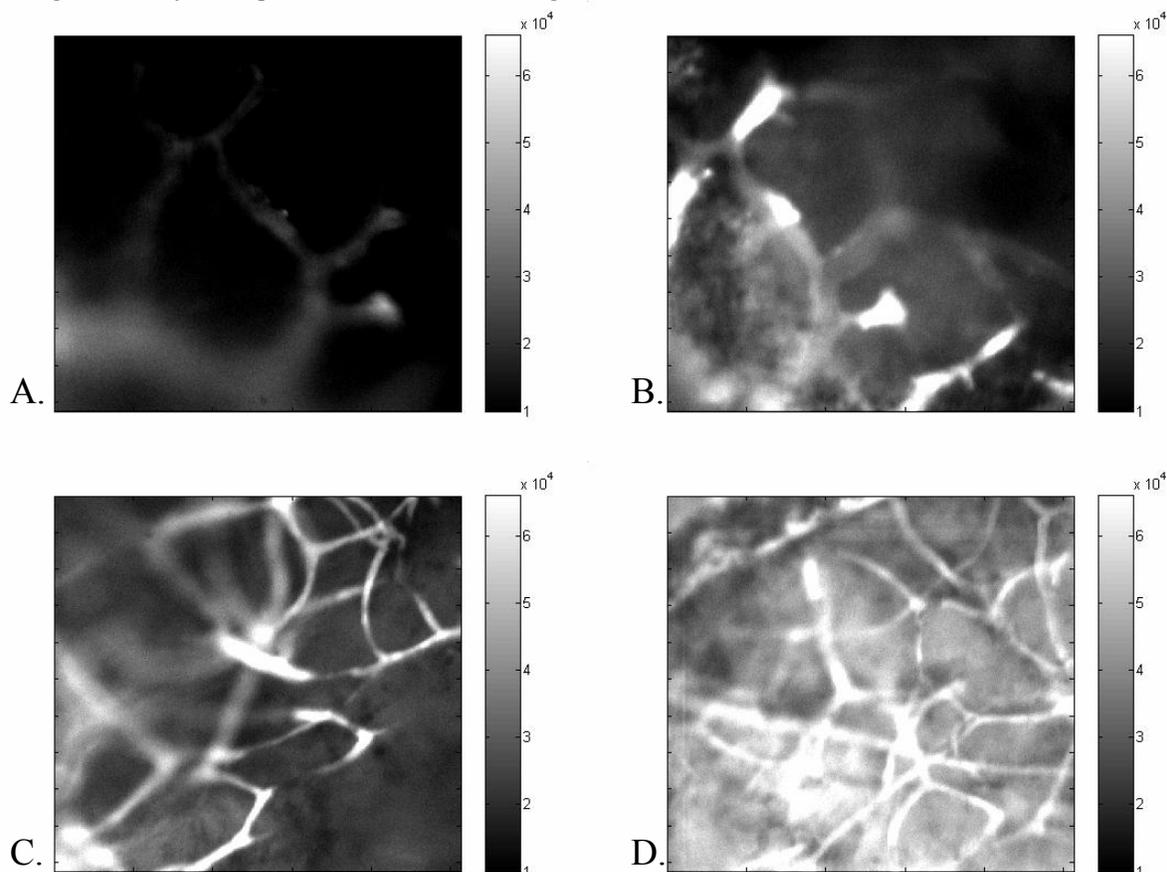
Recent advances in light sources, detectors and other optical imaging technologies coupled with the development of novel optical contrast agents have enabled real-time, high resolution, *in vivo* monitoring of molecular targets, particularly statically anchored molecular targets such as cell surface receptors, in biological systems. With better understanding of tumor biology and enhanced optical imaging capabilities, there has been an increased interest in detecting dynamics of cellular processes using optical technology including cytokines that are actively secreted by the host cells in response to their microenvironment. One of these targets is vascular endothelial growth factor (VEGF), an angiogenic cytokine that is critical for tumor growth and metastasis and has a potential role in limiting the tumoricidal effects following cancer therapies such as radiotherapy and photodynamic therapy (PDT). Our lab has developed a preclinical model in which the post-therapy VEGF upregulation can be controlled by PDT treatment parameters.

VEGF is an angiogenic factor that is critical for vascular permeability, angiogenesis and metastasis (11) in various tumors including PCa (12). It is particularly relevant to cancer treatments such as radiotherapy and PDT because they lead to increased VEGF expression (13-15), potentially contributing to increased metastasis following treatment (16, 17). On a similar note, combination of radiotherapy and PDT with anti-angiogenic treatments has lead to decreased metastasis (13) and increased antitumor effect (18, 19). Monitoring of VEGF expression *in vivo* will lead to valuable insights into the immediate molecular response following combination therapy and its subsequent effect on long-term treatment response. There is limited report of monitoring VEGF expression *in vivo* (20) using radiolabeled VEGF antibodies was used with PET imaging. Functional changes in vascular permeability due to VEGF expression could be monitored by imaging the vascular diffusion of fluorescent macromolecules (21).



**Figure 4.** Images of subcutaneous tumors acquired using the Maestro *in vivo* imaging system. A control mouse that was not injected with RhuVEGFMab-Alexa Fluor 488 conjugate, a mouse treated with 100  $\mu$ L CoCl<sub>2</sub> and injected with rhuVEGFMab-Alexa Fluor 488 and a mouse treated with 100 mL PBS and injected with rhuVEGFMab-Alexa Fluor 488 were placed on stage. (a) shows the reflected white light image of the three mice. Magnified, spectrally-unmixed fluorescence image of Alexa Fluor 488 at the tumor site of (b) the control mouse, (c) CoCl<sub>2</sub>-treated mouse, and (d) PBS-treated mouse are also shown. (e) shows the average fluorescence intensity calculated from the tumors shown in (b), (c) and (d).

In order to monitor the changes in VEGF expression following PDT, we developed a VEGF-specific contrast agent by conjugating an anti-VEGF antibody with fluorescent dye molecules. We tested these contrast agents in a preliminary study using subcutaneous prostate tumor models in immune compromised mice. One of the tumors were treated with subcutaneous injection of cobalt chloride to induce VEGF expression (22). Following intravenous injection of the VEGF-specific contrast agent, we observed approximately a 50% increase in fluorescence intensity in the cobalt chloride-treated tumor compared to the saline-treated tumor (Figure 4). We are planning to apply the molecular specific contrast agent to study the expression of VEGF following PDT.



**Figure 5.** Monitoring changes in vascular permeability using in vivo fluorescence imaging. 200 kDa dextran molecules coated with FITC was injected intravenously into mice that was not treated with PDT ((a), (b)) and that was treated with PDT ((c), (d)). Images were taken from the treatment site following PDT 0 minutes ((a), (c)) and 20 minutes ((b),(d)) following dextran-FITC injection.

Optical imaging tools can also be used to monitor physiological changes following PDT. Changes in vascular permeability have been quantified by injecting fluorescent macromolecules intravenously and monitoring them with a fluorescence microscope (23). Using similar methods, we wanted to monitor changes in vascular permeability following PDT. 200 kDa dextran molecules coated with fluorescein isothiocyanate (FITC) was injected intravenously and the diffusion of these macromolecules were traced using the intravital fluorescence microscope. The acquired images show that there is increased diffusion of the macromolecules from the vasculature into the surrounding tissue in PDT-treated tumors compared to untreated tumors.

## 4. CONCLUSIONS

This review provides an overview of application of molecular imaging in the field of PDT. With the advent of new molecular contrast agents as well as optical detection technologies, the field of optical treatment and diagnosis is rapidly evolving. Optical imaging can be applied to monitor various molecular aspects of PDT, such as photosensitizer delivery, post-therapy changes in molecular expression and tissue physiology. Capabilities to monitor molecular changes will not only help understand the molecular mechanisms of treatment but will also help optimize treatment parameters and improve therapeutic efficacy.

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