New insights into photodynamic therapy of the head and neck
Karakullukçu, Bar

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Chapter 5

Clinical Feasibility of Monitoring m-THPC Mediated Photodynamic Therapy by Means of Fluorescence Differential Path-length Spectroscopy


Baris Karakullukcu,
Stephen Chad Kanick,
Jan Bonne Aans,
Henricus JCM Sterenborg,
I. Bing Tan,
Arjen Amelink, and
Dominic J. Robinson
ABSTRACT

The objective quantitative monitoring of light, oxygen, and photosensitizer is challenging in clinical photodynamic therapy settings. We have previously developed fluorescence differential path-length spectroscopy (FDPS), a technique that utilizes reflectance spectroscopy to monitor microvascular oxygen saturation, blood volume fraction, and vessel diameter, and fluorescence spectroscopy to monitor photosensitizer concentration. In this paper the clinical feasibility of the technique is tested on eight healthy volunteers and on three patients undergoing PDT of oral cavity cancers. Model-based analysis of the measured spectra provide quantitative tissue parameters that are corrected for background tissue absorption, autofluorescence, and the transmission of the optical system; this method allows comparison of intra- and inter-subject parameters. The FDPS correctly estimated the absence of mTHPC in volunteers and detected photobleaching in the areas receiving treatment light in patients undergoing PDT treatment. This study demonstrates the feasibility of monitoring clinical photodynamic therapy treatments using optical spectroscopy.
Chapter 5

1. INTRODUCTION

Photodynamic therapy (PDT) has been gaining acceptance in several branches of medicine, ranging from dermatology to neurosurgery[1-7]. PDT is a targeted treatment modality that involves the administration of (non-thermal) light and a light sensitive drug, termed a photosensitizer, to yield localized tissue destruction with minimal systemic toxicity. When the photosensitizer is activated, light energy is transferred to molecular oxygen, which leads to the formation of reactive oxygen species (ROS). ROS combine with biomolecules, oxidizing them, leading to the destruction of the illuminated tissue by a range of mechanisms that include; direct tumor cell kill, destruction of tumor vasculature and an immune response against tumor cells[8-10].

A number of centers, including our own, are utilizing PDT to treat cancers of the head and neck[1-4]. Careful selection of the patients with regard to complete light access and depth of tumor has yielded encouraging results. The published data report 69-95% complete clinical response to PDT of early oral cavity cancers[1-4]. However regardless of the careful selection criteria and standard treatment technique, a small but significant number of tumors do not respond to PDT. Methods that enable a deeper understanding of the PDT process and bring this understanding down to individual treatment sessions could be of substantial use for the clinician.

There are numerous factors that can influence the response of tissues to PDT. The local efficacy is mediated by the production of ROS and depends on the presence of three components; light, photosensitizer and oxygen[8]. Each must be present in sufficient quantities within the treatment volume[8,11]. Earlier studies have shown inter- and intra-subject variations in parameters such as photosensitizer pharmacokinetics, tissue optical properties and subsequent differences in delivered fluence (rate), and the ability of the local vasculature to provide sufficient oxygen during therapy[12-15]. Each of these variables can be different for individual lesions, patients, and crucially, can be interdependent and change dynamically during, and often as a result of therapy[15-18]. These factors can lead to variations in the PDT dose that is delivered to the target tissue and the surrounding normal tissues and may be the source of treatment failures.

Optical spectroscopy is a promising tool to monitor PDT related variables[13-21]. Reflectance spectroscopy utilizes the dominant absorption bands of hemoglobin in oral mucosal tissue to measure parameters that characterize local vascular physiology, such as microvascular oxygen saturation, and blood volume fraction[13,14,21]. Fluorescence spectroscopy fluorescence attributed to optically active photosensitizing compounds, and can be used to monitor the local photosensitizer concentration and photobleaching during
therapy[16-18]. The combination of monitoring reflectance and fluorescence within the same interrogation volume may also provide additional information on the relationship between photobleaching and local supply of oxygen during PDT. When interpreting these types of signals it is important that the measured parameters are representative of the same volume of tissue, are reproducible, and are comparable.

Our group has developed differential path-length spectroscopy (DPS)[19,20] and florescence differential path-length spectroscopy (FDPS) to provide localized descriptions of tissue vascular physiology and photosensitizer concentrations during clinical PDT. FDPS makes use of two optical fibers one for delivery of light (white light in case of DPS and 652nm excitation light in case of FDPS), and both for collecting light reflected (or emitted) from the tissue. The difference of the two collected signals limits the contribution of long path-length photons to the signal, enabling sampling the small volume of tissue immediately adjacent to the fibers. FDPS has the advantage over other quantitative fluorescence measurements with its capability to deal with large variations in background absorption using a simple correction algorithm. This makes FDPS especially valuable for in vivo photosensitizer fluorescence spectroscopy during PDT, when the background absorption can change significantly. Another advantage of FDPS is that the collection volume can be adjusted to match the relevant dimensions of the application. For absolute fluorescence measurements of photosensitizers it is essential to selectively interrogate the relevant tissue volumes and to avoid averaging drug concentrations over a volume that is either deeper or shallower than the intended sampling location. It is important to note that while FDPS remains dependant on the scattering coefficient of tissue this is expected to have a relatively small influence on the signals collected particularly in tissues of the same type[20].

The aim of the current study was to incorporate this technique in the clinical setting. This manuscript details the development of a FDPS system, to be utilized in patients undergoing meta-tetrahydroxyphenylchlorin (mTHPC) mediated-PDT for oral cavity carcinomas, without interrupting the clinical protocol. Processing of the data obtained, to yield quantitative data that can be compared both intra-subject (measurements performed in the same patient), and inter-subject (measurements from different patients) is explained in detail. The reliability and reproducibility of these measurements and future clinical applications are discussed.

2. MATERIALS AND METHODS

2.1. STUDY POPULATION AND CLINICAL METHODS

This clinical pilot study is carried on 8 healthy volunteers and 3 patients with early stage cancers of the oral cavity without regional metastasis (T1N0 by AJCC staging), who are
undergoing PDT. All patients had histologically proven squamous cell carcinoma. The depth of the tumors have been measured and confirmed to be less than 5 mm by ultrasonography (US). Informed consent has been obtained from patients in accordance with institutional guidelines as approved by the institutional review board of Netherlands Cancer Institute. mTHPC was injected to a proximal vein at a dose of 0.15 mg/kg as outpatient procedure. After an interval of 4 days the patients received PDT in an operation room setting, under general anaesthesia. FDPS measurements were first performed immediately before therapeutic illumination. Measurements were taken from at least three locations on the center of the tumor, three locations on the adjacent normal appearing mucosa that received treatment light as oncologic safety margin and three locations in the oral cavity, distant from the tumor, located under shielding (therefore not receiving treatment light). The oral cavity mucosa that was not intended for PDT is shielded from the treatment light by green operation drapes or black shielding wax. Tumors were treated with light from diode laser 652nm (Biolitec, Germany) with an oncologic margin of at least 5 mm around the macroscopic tumor using at a irradiance of 100 mWcm$^{-2}$ to a radiant exposure of 20 Jcm$^2$. Immediately after the therapeutic illumination FDPS measurements are repeated at the same sites as before the illumination.

FDPS measurements from the volunteers are used to determine the basis spectra for tissue auto fluorescence; FDPS measurements from the patients are used to visualize the effects of PDT.

2.2. FDPS SETUP

The device setup for optical measurements utilized in this study is an adaptation of the FDPS device described previously[19,20]. Figure 1 shows a schematic of the device setup. The FDPS probe (Configured Bifurcated Fiber ZFQ-13177, Ocean Optics, Duiven, NL) contains two 800 µm diameter optical fibers; one fiber is used for delivery and collection of light (dc) and the adjacent fiber is used for light collection only (c). The distal end of the probe was polished under an angle of 15 degrees to minimize specular reflections at the probe-tissue interface during measurements. The 800 µm dc fiber is coupled to a 4 x 150 µm quadrifurcated fiber. The first arm of this quadrifurcated fiber is connected to a tungsten halogen lamp (HL-2000, Ocean Optics, Duiven, NL). The second arm is led to a 652 nm 0.5 W channel of a medical diode laser unit (Cerelas 4 x 1 mW 532 ± 1 nm + 652 ± 3 nm 0.5 W CW, Biolitec AG, Jena, DE) routinely used for clinical PDT with m-THPC set to deliver 2 mW at the probe distal end. The third arm is connected to a 532 nm 1 mW channel of the laser unit. The fourth arm is led to a spectrometer (S2000, Ocean Optics, Duiven, NL). The bandwidth of the 652 nm laser was narrowed with a custom built filter unit combining a
660 nm long-wave-pass interference filter (3RD660LP, Omega Optical, Brattleboro, USA) and a 660 nm short-wave-pass interference filter (3RD660SP, Omega Optical, Brattleboro, USA). The filter unit collimates light coupled in and directs the beam through two optical filter mounts that can be angled with respect to the beam. This allows for changing the angle of incidence of the collimated beam on the mounted interference filters which effectively shifts the filter spectral transmission characteristics to the blue. The 800 µm c fiber is leading to a second spectrometer (S2000, Ocean Optics, Duiven, NL). A second filter unit containing a 658 nm notch filter (NF03-658E-25 StopLine, Semrock, Rochester, USA) is used to filter the 652 nm light from the fibers leading to the spectrometers. With the filters are carefully tuned almost all excitation light is filtered out allowing the system to record both the relatively weak fluorescence as well as the important spectral features needed for DPS. Reflectance and fluorescence measurements were made consecutively by using shutters for each light source allowing selective usage of the light sources for each measurement. A custom made LabView (v7.1, National Instruments, Woerden, NL) program was designed to control shutters and spectrometers via a notebook computer. Throughout this manuscript, the difference between the white-light reflectance measured by the dc- and c-fibers is termed the differential reflectance (DR). Similarly, the difference between the fluorescence collected by the dc- and c-fibers is termed differential fluorescence (DF). We note that $I$, $J$, and $F$ are wavelength dependent parameters in subsequent sections.

Figure 1. Skematic of the FDPS device setup. See text for details.
2.3. FDPS CALIBRATION

Calibration of the DR and DF signals is described in detail previously [19,20], and is outlined briefly here. Calibration of DRa signals involves measurement of white-light spectra from black and white Spectralon standards (Labsphere SRS-99 and SRS-02) in air, and a separate measurement in a water-filled dark container. This is given as,

\[
DR = c \left[ \frac{(I^R_R - I^R_{\text{water}})}{(I^R_{\text{white}} - I^R_{\text{black}})} - \frac{J^R_R}{(J^R_{\text{white}} - J^R_{\text{black}})} \right]
\]

Here \( I^R \) and \( J^R \) represent the white-light intensity collected by the dc-fiber and c-fiber, respectively. The \((I^R_R - I^R_{\text{water}})\) difference corrects the intensity collected by the dc-fiber for imperfections on the probe face that introduce to internal reflections[22]. Fiber transmission characteristics and variations in lamp output are accounted for by dividing the \( I^R \) and \( J^R \) signals by their differences between the measurements on white and black Spectralon, given as \((I^R_{\text{white}} - I^R_{\text{black}})\) and \((J^R_{\text{white}} - J^R_{\text{black}})\). The calibration constant \( c \) depends on the distance between the probe tips and the Spectralon.

Calibration of the DF signal requires application of the system transmission efficiency profile to transform the fluorescence intensities returned by the spectrophotometer into fluorescence quantities independent of fiber transport properties and spectrophotometer sensitivities. This calculation is given as:

\[
I^F_{\text{sample}} = F^F_{\text{sample}} \cdot T^F_{\text{dc}}
\]

\[
J^F_{\text{sample}} = F^F_{\text{sample}} \cdot T^F_{c}
\]

Here \( I^F_{\text{sample}} \) and \( J^F_{\text{sample}} \) represent the total fluorescence intensity returned by the spectrophotometer from measurements from the dc-fiber and c-fiber, respectively. These quantities are described as the product of the number of photons entering the fibers \((F^F_{\text{sample}} \text{ and } F^F_{\text{sample}})\) and the transmission function describing the efficiency of photons travelling from the tip of the fiber to the spectrophotometer \((T^F_{\text{dc}}\text{ and } T^F_{c})\); this calculation is appropriate for the dc- and c-fibers, respectively. With the beam shaping and notch filter sets optimised to reject scattered laser light most efficiently, the wavelength dependence of the transmission functions \( T^F_{\text{dc}} \) and \( T^F_{c} \) were measured using a calibrated white light source (Ocean Optics HL-2000-CAL, FL, USA) for which the output of the lamp is well-known; characterization of this curve has been described in detail previously[23]. Variations in the absolute value of the \( T^F_{\text{dc}} \) efficiency function between different measurement sessions were corrected using the ratio of reflectance intensity measured with the dc-fiber using
a laser source at 532 nm (coupled into the laser source arm of the FDPS setup) on a highly scattering solid phantom to the power of that laser output at the end of the dc-fiber. Independent assessment of variations in the absolute value of the $T_c$ efficiency was determined using reflectance measurements on white and black Spectralon to yield a factor

\[ r_c = \left( \frac{J_{\text{white}}^R - J_{\text{black}}^R}{J_{\text{white}}^R - J_{\text{black}}^R} \right) \]

evaluated at 532 nm. Day-to-day variations in excitation laser output from the distal end of the dc-fiber are measured measuring output power of the laser exiting the distal end of the dc-fiber by a power meter connected to an integrating sphere, given as $P_{\text{cal}}$. The resulting calibrated DF signal can be expressed in units of photon counts/ms/mW, and is calculated as:

\[
DF_{\text{Mears}} = F_{\text{sample}}^{dc} - F_{\text{sample}}^{c} = \frac{1}{P_{\text{cal}}} \left( \frac{J_{\text{sample}}^F}{T_{\text{dc}}} - \frac{J_{\text{sample}}^F}{T_{\text{c}} r_c} \right)
\]

### 2.4. FDPS DATA ANALYSIS

The DR signal resulting from Equation (1) was fitted according to the following empirical model:

\[
DR_{\text{Model}} = a \left[ f \frac{\lambda^b}{\lambda_0} + (1 - f) \frac{\lambda^a}{\lambda_0} \right] \exp(-\mu_a \cdot <L>)
\]

The term in brackets represents a background scattering model composed of Mie and Rayleigh components. The fractional contributions of Mie and Rayleigh components are estimated by fitting the parameter $f$ (which occupied values in the range 0-1), and the respective wavelength dependencies of the components are given as $\lambda^b$ and $\lambda^a$, where $b$ is a fitted parameter. The fitted parameter $a$ corrects for changes in the absolute amplitude of the measured DR signal; the value of $a$ is dependent on the distance between probe and Spectralon during calibration measurements and does not inform estimates of tissue scattering properties. The effect of absorption on the DR signal is modelled by applying a Beer-Lambert law to the background scattering model. The absorption coefficient within the oral cavity is given as:

\[
\mu_a = \rho \cdot \left( StO_2 \cdot \mu_a^{HBO_2} (\lambda) + (1 - StO_2) \cdot \mu_a^{Hb} (\lambda) \right) \cdot C_{\text{corr}} (\lambda) + Bil \cdot \mu_a^{Bil}
\]

where $\rho$ is the blood volume fraction (BVF); $StO_2$ is the microvascular oxygen saturation, $\mu_a^{HBO_2}$ and $\mu_a^{Hb}$ are the specific absorption coefficients of fully oxygenated and deoxygenated blood\(^{23}\), respectively, and $C_{\text{corr}}$ is a correction factor that depends on vessel diameter ($d_v$) and accounts for the inhomogeneous distribution of blood in tissue\(^{24}\), and is given as:
where \( \mu_{a,bl} \) is contribution of the absorption of blood to as in Equation (6). \( \text{Bil} \) is the concentration of bilirubin and the contribution of bilirubin to the tissue absorption is calculated by using the specific absorption coefficient \( \mu_a^{\text{Bil}} \) [25]. The DPS photon path length given as \( \langle L \rangle \), experienced in tissues of the oral cavity is insensitive to effects of the scattering coefficient (as it is expected to fall within the range of 5-50 mm\(^{-1}\) for wavelengths in the 300-900nm range), but \( \langle L \rangle \) can be affected by the absorption coefficient (especially for the probe diameter utilized in this study). We accounted for the dependence of \( \langle L \rangle \) on \( \mu_a \) by using an empirical expression described previously by Kaspers et al[26]. The fitting procedure was conducted using a Matlab code to perform a Levenberg-Marquardt least squares fitting routine using the lsqnonlin function within the Matlab optimization toolbox, this procedure utilizes the standard deviations of the binned data points as weight factors. The fitting procedure estimated mean values and confidence intervals for \( a_1, b, \rho, StO_2, d \), \( \text{Bil} \), using a method described previously[27].

The \( \text{DF} \) signal resulting from Equation (4) was corrected for absorption effects by multiplying \( \text{DF} \) by the ratio of the DR at the excitation wavelength without and with absorption present, as follows:

\[
\text{DF} = \text{DF}_{\text{Mean}} \frac{DR_x}{DR_x(\mu_{a,x})}
\]

Where \( DR_x \) and \( DR_x(\mu_{a,x}) \) are the differential reflectance signals at the excitation wavelength measured with and without background absorber present, respectively. \( DR_x(\mu_{a,x}) \) was calculated from the model fit resulting from Equation (5), while \( DR_x \) was estimated by the background scattering model (the term appearing within brackets) within the same Equation. It should be noted that the \( \text{DF} \) signal resulting from Equation (8) is not corrected for effects of scattering on the collected signal. The absorption corrected \( \text{DF} \) signal was analyzed as a linear combination of basis spectra representing tissue autofluorescence, m-THPC fluorescence, and background fluorescence that is attributable to fluorescence of the optical components within the FDPS system. We note that the magnitude of this system background fluorescence is dependant on the scattering coefficient of the tissue within the interrogation volume and cannot be simply measured and subtracted. The model estimate of \( \text{DF} \) is given as,
Feasibility of clinical FDPS in head and neck PDT

\[
DF_{\text{Model}} = b_{\text{mTHPC}} \xi_{\text{mTHPC}} + b_{\text{system}} \xi_{\text{system}} + \xi_{\text{auto}} \tag{9}
\]

where, \( \xi_{\text{auto}} = c_1 \left( \frac{\lambda}{\lambda_o} \right)^3 + c_2 \left( \frac{\lambda}{\lambda_o} \right)^2 + c_3 \left( \frac{\lambda}{\lambda_o} \right) \tag{10} \)

Here, \( (\xi_{\text{system}}, \xi_{\text{mTHPC}}) \) represent the basis spectra and \( (b_{\text{system}}, b_{\text{mTHPC}}) \) represent the contribution of fluorescence attributable to the components within the FDPS system (e.g. the filter set) and m-THPC, respectively. The lineshape of \( \xi_{\text{system}} \) was identified by DF measurements on white Spectralon, which does not fluoresce. The lineshape of \( \xi_{\text{mTHPC}} \) was calculated from DF spectra acquired in vivo corrected for fluorescence attributable to the endogenous tissue or system components, and was determined from the average lineshape extracted from clinical DF measurements on multiple patients obtained before and after treatment illumination. This paper assumes a linear proportionality between \( \xi_{\text{mTHPC}} \) and mTHPC concentration. The contribution of tissue autofluorescence to DF is given by \( \xi_{\text{auto}} \) and can be characterized by a third-order polynomial; this functionality was identified from DF measurements in the oral cavity with no m-THPC present and was corrected to account for the fluorescence from system components. The fitting procedure was again conducted using a Matlab code to perform a Levenberg-Marquardt least squares fitting routine that uses the standard deviations of the binned data points as weight factors; the fitting procedure estimated mean values and confidence intervals for \( b_{\text{mTHPC}}, b_{\text{system}}, c_1, c_2, c_3 \).

High quality fitting was achieved in over 95% of measurements, also in the case of relatively low blood volumes. Spectra that did not fit were excluded based on the presence of observable features in the residual between the model fit and the data[27]. The poor quality of some individual measurements was due to patient and/or probe movement and incomplete contact between the probe and the oral mucosa.

3. RESULTS

3.1. FDPS SYSTEM TRANSMISSION

Figure 2a shows a representative \( I^f \) spectrum of the dc-fiber measured in vivo superficially on the center of an oral cavity cancer, 4 days after mTHPC injection, immediately before illumination. The markers on the figure represent mean of 7 binned data points (each bin spans ~2 nm), with the standard deviation calculated from data points within each bin are representative of noise within the spectrum. This spectrum represents the wavelength-dependent fluorescence returned by the spectrograph following excitation of tissue with 652 nm light, with the signal expressed per integration time and excitation...
light power. Figure 2b shows the $T_{dc}$ of the dc-fiber, which describes the wavelength-dependent efficiency of photons entering the fiber tip that are eventually returned by the spectrograph; this curve characterizes wavelength-dependent fiber transmission effects and spectrophotometer sensitivities. Inspection of these data clearly show the effect of the notch filter on the measured fluorescence, with zero photons entering the fiber transmitted to the spectrophotometer at the excitation wavelength which is in the notched wavelength region centred on 652 nm. Using Equation (2) it is possible to calculate $F_{dc}$, which represents fluorescence independent of system transmission effects; this is shown in Figure 2c.

**Figure 2.** a) A representative fluorescence spectrum $[I']$ of the dc-fiber measured at the center of an oral cavity cancer, 4 days after mTHPC injection, immediately before illumination. The markers on the figure represent mean of 7 binned data points (each bin spans ~2 nm), with the standard deviation calculated from data points within each bin are representative of noise within the spectrum. b) Figure 2b shows the transmission function $[T']$ of the dc-fiber, which describe the wavelength-dependent efficiency of photons entering the fiber tip that are eventually returned by the spectrograph. The effect of the notch filter is visible around 652 nm. c) Calculated fluorescence $[F_{dc}]$ independent of system transmission effects

### 3.2. BASIS SPECTRA

Inspection of the representative $F_{dc}$ spectrum shows that a narrow region of interest can be used to characterize m-THPC fluorescence, located to the right of the notch filter. Analysis of fluorescence in this region required specification of basis spectra for both system fluorescence and mTHPC.

The FDPS setup utilized in this study contains a custom designed notch-filter set to prevent excitation light from entering the spectrophotometer. Figure 3a shows a characteristic wavelength-dependent fluorescence signal attributable to system components; these data represent the basis spectrum $[\phi_{system}]$. This signal was observed as a component in all measurements of tissue, and its respective contribution to the collected signal was estimated from the application of Equation 9.
Fluorescence attributable to mTHPC requires accurate specification of the wavelength-dependent fluorescence spectrum emitted in the clinically relevant in vivo environment. This study characterized the mTHPC basis spectra, $\xi_{mTHPC}$, by analyzing the difference in DF spectra at a single location measured before and after treatment illumination after correction for both system and auto-fluorescence. The introduction of illumination light was attributable to mTHPC photobleaching. This normalized differential signal is shown in Figure 3b. The quality of the $\xi_{mTHPC}$ obtained in this manner was confirmed by inspecting residuals between measured and model-fits of DF for features.

3.3. MEASUREMENT OF TISSUE AUTOFLUORESCENCE

Autofluorescence contributes to the DF. FDPS on oral mucosa of healthy volunteers, who did not receive mTHPC, demonstrates this phenomenon efficiently. Figure 4a and 4b show paired FDPS measurements of DR and DF on the floor of mouth of such a volunteer. The DR spectrum shows a good model fit, with the residual showing no features. The DF spectrum shows the composition of the measured signal using $\xi_{mTHPC}$, $\xi_{system}$, and $\xi_{autofluor}$. The model fit shows no residual features. The model fit detects no fluorescence attributable to mTHPC, which is consistent with the fluorophore content of the tissue; the fluorescence detected is solely attributed to system fluorescence and autofluorescence by the fit.

**Figure 3.** a) Wavelength-dependent fluorescence signal attributable to system components. These data represent the basis spectrum $\xi_{system}$. b) The corrected differential fluorescence (DF) of mTHPC.
Figure 4. 4a and 4b show representative paired DR and DF on the floor of a volunteer, demonstrating autofluorescence. The DR spectrum shows good model fit, with residual showing no features. The DF spectrum (4b) shows the composition of the measured signal. The model fit detects no fluorescence attributable to mTHPC.

3.4. FDPS DURING MTHPC-PDT IN THE ORAL CAVITY

Figure 5 shows representative FDPS measurements on a patient undergoing m-THPC mediated PDT. Measurements show representative DF and DR measurements performed at the center of the tumor. Figures 5a and 5b show DR and DF measured before illumination, figures 5c and 5d show the corresponding measurements after illumination. The DF spectrum in Figure 5b shows fluorescence attributable to m-THPC; the corresponding post-treatment DF spectrum (Figure 5d) measured following therapeutic illumination shows a substantial decrease in m-THPC fluorescence as a result of photobleaching during therapy. Tables 1 and 2 show data acquired from FDPS measurements made immediately before and after PDT in 3 patients. Measurements of vascular physiology (microvascular saturation, BVF, and vessel diameter) and mTHPC fluorescence are reported as the average of 3 repeated measurements from each inspection site (tumor center, margin, and normal oral mucosa). The standard deviation shown is indicative of the relative spatial variation within a single site. While it is not the purpose of the present study to assess the statistical significance of differences between sites and patients, the micro-vascular saturation varied from 71 to 99% but did not show any clear trends between sites or patients. The blood volume fraction varied from 0.4 to 4.6% where there was a trend for a lower average blood volume measured in tumor center compared to tumor margin and normal mucosa. It is noteworthy that estimates of physiological parameters from sites with low blood volumes showed larger confidence intervals than sites with high blood volumes. Table 2 shows the corresponding average mTHPC fluorescence measured at each site in each patient before and after PDT. In the 3 patients investigated in the present study the average standard
deviation among measurements made at individual sites was approximately 17% of the mTHPC fluorescence before therapy. Although not significant, the tumor center has higher average mTHPC fluorescence than margin and normal mucosa. As expected, there is significant photobleaching after therapy at sites that were illuminated. The percentage decreases in mTHPC fluorescence at the tumor center were 91%, 80%, and 75%, for patients 1, 2 and 3 respectively. The illuminated margins also showed photobleaching (56%, 48%, and 68%), while the shielded normal mucosa did not show any significant decrease in mTHPC fluorescence.

**Figure 5.** 5a and 5b, show representative DR and DF measured at the center of an oral cavity cancer 4 days after mTHPC injection, before therapeutic illumination; figures 5c and 5d, show the measurements at the same site after illumination. The fluorescence attributable to m-THPC shows a substantial decrease from figure 5b to figure 5d, demonstrating photobleaching.

**4. DISCUSSION**

Objective quantitative data about changes in vascular physiology and photosensitizer concentration during the PDT process would be helpful to define the changes occurring within the treated tissue volume. FDPS gives insight into two of the three components of
PDT: oxygen and photosensitizer. Previous experimental settings have confirmed the validity of the method[13,19,20,28].

FDPS is a method that corrects for the significant absorption variations due to changing blood composition during PDT. DR is scattering independent due to the small path-length of the photons that travel through the tissue at the wavelength range measured. However, FDPS is not scattering independent. Earlier studies have shown that the relative narrow wavelength range of the examined spectra limits the effects of scattering[20]. The low standard deviation of fluorescence measured (Table 2), demonstrates that the scattering dependency should not cause substantial variations in measurements of biological tissue, especially when considering measurements of the same tissue-type.

Determining the basis spectra from the oral mucosa of healthy volunteers enabled us to effectively account for the tissue autofluorescence. As can be seen on Figure 4b the model correctly detects the absence of mTHPC in the oral mucosa of a healthy volunteer. The intra-subject comparability enables us to compare photosensitizer concentration, and oxygen related physiologic parameters in different tissues, such as the tumor, normal mucosa. The quantitative nature of the measurements allows us to compare the changes in vascular physiology and photosensitizer concentration at different time points during PDT, giving us reliable information about the ongoing processes that are occurring.

All individual measurements showed photobleaching in every site examined in the treatment field. The percentage average photobleaching in illuminated tumor sites were 90%, 80%, and 75%, for patients 1, 2, and 3, respectively. The illuminated margins also showed photobleaching as expected (56%, 48%, 68%, for patients 1, 2, and 3 respectively). The photobleaching seems of somewhat less than at the center of the tumor. There could be a number of reasons for this variation, from light distribution to differences in physiology of tumor and normal mucosa. The interpretation of this difference (if it really exists), requires more data collection.

The sites that did not receive treatment light and are protected by shielding did not show any photobleaching with minimal variations probably due to inspector related variation of the measured site. These initial results are able to demonstrate the photodynamic therapy effect on the photosensitizer in every measurement that is taken.

Table 1 summarizes the oxygen and circulation related parameters measured in this pilot study. There is considerable variation in the physiological parameters measured. The variation is representative of the actual situation. This variation can be explained by the small sampling volume and variations in the histopathological composition of the tumor.
and the oral mucosa. For example the thick keratin layer of an oral cancer, or total absence of it in case of an ulcer, would have considerable variations in blood content. If a large blood vessel is located within the sampling volume, the BVF would be higher, as well as the vessel diameter. Taking several measurements and accepting the average as representative, could overcome this potential hindrance. At first glance there is not a substantial change in physiological parameters measured before and after PDT. There was a trend for a higher average blood volume measured in tumor center compared to the tumor margin and in normal tissue. However the data set is too small to derive any conclusions. If there are any changes due to PDT, these can be detected once sufficient data from future PDT sessions are accumulated.

DR and DF should always be evaluated as paired data. For example DR spectra can detect the presence of a hematoma under the mucosa, through high BVF and vessel diameter values, which has two clinical implications. Firstly the measured fluorescence could be of the blood rather than the treated tumor, which has no clinical relevance. Secondly the haemoglobin in the hematoma, if present before PDT can act as a filter absorbing the therapeutic laser light and preventing activation of the photosensitizer. Combining DR and DF fits can give us a better understanding of the inspected site.

The FDPS setup described in this manuscript was easily applicable both in operating theatre and as a bedside procedure. Individual measurements each took 5-10 seconds each to perform and therefore did not add significantly to the anesthesia time. The procedure did not cause any discomfort to the patient. The hand-held probe with the dc and c fibers was easy to manipulate by the clinician treating the patient. The minimal burden of employing FDPS in clinical setting, gives us an opportunity to routinely employ this technique to gather information about PDT procedures.

The FDPS technique can also be applied to bulkier tumors receiving interstitial PDT, via implantable probes. This would enable us to monitor the whole PDT process by taking measurements at several time points during the PDT rather than two measurements of before and after PDT. This would be of particular interest to profile the dynamic PDT process in vivo.

As the data set builds up we will have more objective insight to the processes occurring during PDT in vivo. Obtaining information over a broad clinical data set has the potential to not only define observations associated with the standard (successful) administration of PDT treatment, but also identify and detect aberrations from the standard process. This approach can give us ideas about the reasons of treatment failures; whether it would be treatment site or treatment technique related.
5. CONCLUSIONS

The results presented in this paper indicate that FDPS can safely be utilized in the clinical setting to obtain quantitative and reproducible parameters that describe tissue microvascular saturation, blood volume fraction, average vessel diameter and photosensitizer fluorescence. The FDPS correctly estimated the absence of mTHPC in volunteers and detected photobleaching in the areas receiving treatment light in patients undergoing PDT treatment. These data show the feasibility and potential clinical utility of using FDPS to monitor clinical PDT treatments.
6. REFERENCES


