System for Fluorescence Quantification of Thin Tissue Layers
Guided by High Frequency Ultrasound

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ABSTRACT

A system is presented which has been developed for dermatological applications with the need to quantify levels of protoporphyrin IX (PpIX) in diagnosis or therapy. The design was to couple fluorescence sampling onto a high frequency ultrasound system and take multiple optical source-detector samples of the tissue of the fluorescence and transmission signals. The intensity values can then be used to estimate and image the PpIX levels present in tissue samples. The system design, calibration, and initial testing in tissue phantoms are demonstrated here. The component design has been modular and allows easy implementation as a kit which can be assembled from basic components. The control software is more elaborate and provides a seamless way to go from system start up through to fluorescence quantification of PpIX concentrations.

1. INTRODUCTION

The goal of this study has been the basic development of a system which could quantify protoporphyrin IX (PpIX) concentration levels in skin tumor tissue through direct measurement of the fluorescence coming from the tissue, but with the goal of allowing the use of imaging at the same time to guide the interpretation of the signal (1-3). Assuming that fluorescence can be linearly related to the concentration of the fluorophore, direct measurements can reasonably be used to quantify PpIX, but the effect of scatter and absorption within the tissue and the effects of tissue layers with varying amounts of PpIX all contribute to the relationship of signal to concentration being non-linear. The most advanced approach to solving this problem is through the use of image-guided fluorescence tomography (4) in a geometry which allows visualization of the skin layers and tumor tissue. For this particular application, high frequency ultrasound (HFUS) was chosen as the optimal visualization technology, and a custom system was designed to allow quantification of the PpIX from the skin layers.

Skin cancer is known to produce PpIX when applied with aminolevulinic acid (ALA), either topically or through systemic administration (5-7). The use of ALA and ALA-ester mediated photodynamic therapy has been growing in use and looks promising for superficial lesions, but the concern for deeper basal cell carcinoma has been the inability to...
reliably produce PpIX at deep locations, or even know if the PpIX was being produced. There are methods recently
developed such as differentiation therapy [8] which have the potential to produce more PpIX at deeper depths, but need to
be studied in a systematic clinical trial. The system developed here is diagnostic and could be applied to human use
once calibrated and tested in phantoms as is reported here.

The design of such a system requires the use of sub-surface tomography, as the signal from different tissue layers
must be differentiated. Doing this without image-guidance has been attempted but the accuracy is not well estabished.
Since the advent of image-guided fluorescence localization, it has become quite realistic to think about doing this under
the real time guidance of high frequency ultrasound. HFUS is an ideal choice for skin and skin tumors because of its
ability to resolve layers to sub millimeter thicknesses, but also allowing penetration of up to 1 cm into tissues, when
tumors wind up being thicker. While no HFUS scanners are clinical approved, they can be used in studies when
approved by the local IRB, and the coupling with optical fluorescence is readily achieved, as is discussed here.

The fluorescence recovery is done through diffusion modeling of the light transport [9], and fitting the calculated
values to the measured values. The most robust data set is also one which does not just measure fluorescence intensity
between fibers, but measured the fluorescence and the transmitted excitation signal as a ratio, which winds up canceling
out many artifactual errors due to fiber coupling and transmission efficiency. The system here was specifically
designed to have this feature, and also provide automated calibration to the simulation fitting process. The system
specifications and performance are presented and analyzed here.

2. METHODS AND MATERIALS

The system (schematic shown in Figure 1) has been assembled from various commercially available products and has
been specifically designed to be as modular as possible. The system has four source channels and five detection
channels. These channels are alternating as shown in Figure 1. The entire control of the system is automated through
a LabVIEW interface.

Each detection channel is comprised of a single spectrometer (OceanOptics Inc., USB 2000+), an inline filter
holder (OceanOptics Inc., FHS-UV) which holds a 650-nm long-pass filter (Thor Labs Inc., FEL0650), a focusing
gradient index (GRIN) lens (Newport, LGI630-6), and various fiberoptic cables for connections. The five channels
connect to a computer via a USB hub. The source (Thor Labs Inc., MCSL635) has 4 independent 635-nm lasers which
are each controlled by the computer through two USB data acquisition cards (National Instruments, NI-6009). The
source channels are attached to GRIN lenses and fiberoptic cables.

The source and detector channels are mounted to the high-frequency ultrasound transducer (VisualSonics,
RMV-708) using a custom-built removable piece. The ultrasound system is a VisualSonics Vevo 770 high-resolution
imaging system. The optical data and ultrasound image can be collected simultaneously and independent of each other.
The optical data set is then calibrated and reconstructed using the anatomical data obtained from the ultrasound image.

The data calibration procedure is shown visually by the flowchart in Figure 2. The entire process is automatically
done through LabVIEW with the output being twenty fluorescence/transmission ratios (one number for each
source/detector pair) in counts/second. When data is collected, a background signal is taken for each spectrometer and
saved as the dark counts for the specific experiment. These dark counts represent the noise baseline of each
spectrometer. This data is subtracted from the measured intensity.

Since each spectrometer has a different sensitivity, the data is normalized by an experimentally determined
sensitivity factor to correct for this. The data is then spectrally fitted to separate the fluorescence from the bleed-through signal. After performing spectral fitting, the difference in filter ODs needs to be addressed. The transmission data from each signal is normalized by the OD of each filter using previous experimental knowledge.

Figure 1. System schematic showing components and probe geometry, with data acquisition through a USB hub to 5 compact spectrometers, which have light filtered as delivered through pick up fibers from the probe tip. The light is delivered from a switchable laser source, with 4 possible locations.

Next, since the integration times are adjusted automatically to ensure enough transmission data is present for detectors far away from the source that is on, the data for each channel is divided by its respective integration time. Finally, the sum of the fluorescence data is divided by the sum of the transmission data. This normalizes the fluorescence measurement to the transmission data, and calculates the fluorescence/transmission ratios. These ratios are output to a file and saved. The calibration procedure and saving routine has been fully tested and implemented using the LabVIEW software.

Figure 2. Flowchart of calibration procedure
3. RESULTS

Using the system, we have measured the F/T ratios of several serial dilutions. Figure 3 shows a typical raw data spectrum acquired at each spectrometer. The spectra shown are of a solution with a concentration of 10 ug/mL PPIX mixed with 1% Intralipid. The data was taken when laser 1 on, and lasers 2-4 off. The intensity measured at detectors 1 and 2 is on the same order of magnitude as expected because laser 1 is directly in between them. With the exception of spectrometers 3 and 4, the signals then decrease, and it is obvious that spectrometer 5 has the lowest signal because it is the detector farthest away from the laser source that was on. This discrepancy is taken care of by the data calibration procedure.

Figure 3. Example spectra of PPIX mixed with Intralipid from each spectrometer with laser 1 turned on. Going from the spectrometer closes to the laser towards the spectrometer that his most distant from the laser, the overall intensity decreases due to attenuation (top left to bottom). a) Spectrometer 1, b) Spectrometer 2, c) Spectrometer 3, d) Spectrometer 4, e) Spectrometer 5
Early on in the development of the system, it was necessary to determine the reliability of the system. Using a solid phantom with known properties, \( \mu_a = 0.0076, \mu_s' = 0.77 \).

From equation 1, we get the value of the effective attenuation coefficient, \( \mu_{\text{eff}} \).

\[
\mu_{\text{eff}} = (3 \times \mu_a \times \mu_s')^{1/2} \quad \text{(Eq. 1)}
\]

This leads to:

\[\mu_{\text{eff}} = 0.1325\]

In order to measure the \( \mu_{\text{eff}} \) of the phantom using the system, we measure the fluorescence signal with one laser source on and plot it versus the distance the detector is from the laser. The slope of this line should approximately be the value of \( \mu_{\text{eff}} \). As shown in Figure 4, the results of the test correlated quite well to the calculated \( \mu_{\text{eff}} \).

![Figure 4](http://spiedigitallibrary.org/)

**Figure 4.** The slope of the linearly decreasing signal with distance away from the laser source allows the approximation of \( \mu_{\text{eff}} \) based upon the assumption that the sample is diffuse.

As previously mentioned in the discussion on the calibration procedure, a technique called spectral fitting is used to separate the laser bleed through signal from the actual PpIX fluorescence. Even with the 650 nm long-pass filters in line with the detection channel, there is bleed through. Spectral fitting, when used with filtering, has been shown to increase the accuracy of concentration measurement \((10)\). Typically when using serial dilutions with different PpIX concentrations as our tissue phantoms, a linearly increasing trend is observed as the concentration increases. At lower concentrations, the signal tends to level out since the signal is mostly noise and laser bleed through. When the spectral fitting is performed, the linear detection range of the system is increased which allows us to measure lower
concentrations of PpIX. Figure 5 shows how this linear range is extended. This data is from a set of serial dilutions ranging from 10 ug/ml PpIX in intralipid down to 0.01 ug/ml. The data shown was gathered on detector 2 when source 1 was on. In all of the graphs, the blue function is the data before spectral fitting and the pink function is the data after spectral fitting.

**Figure 5.** The above plots show the serial dilution data before and after spectral fitting for spectrometer 2.

### 4. DISCUSSION

As previously stated, the quantification of PPIX concentration in skin tissue can be determined by measuring the fluorescence coming from the skin. Since skin cancer has increased production of PpIX when ALA is topically applied, it is reasonable to measure PpIX concentrations around and inside the tumor for the purpose of localization. The system proposed and implemented has been designed for this purpose. Using anatomical information from high frequency ultrasound images, a tomographic reconstruction can be performed to localize the skin tumor for treatment. Initial experimental data from liquid and solid phantoms taken with the system has been promising, and the utility of this system will continue to be investigated in the near future.

So far, the system has proved to be accurate and has performed as expected. The data calibration has allowed us to greatly increase the accuracy. As seen in the discussion surrounding Figure 4, the system was used to approximate the $\mu_{\text{eff}}$ of a solid phantom accurately. In the near future, we will begin work on some animal experiments with hopefully the same success that we’ve had with phantoms.

### 5. ACKNOWLEDGEMENTS

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6. REFERENCES


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