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ABSTRACT

Aminolevulinic acid (ALA)-induced Protoporphyrin IX (PpIX)-based photodynamic therapy (PDT) is an effective treatment for skin cancers including basal cell carcinoma (BCC). Topically applied ALA promotes PpIX production preferentially in tumors, and many strategies have been developed to increase PpIX contrast in tumors, especially in deeper tumors. Deep tumors respond poorly to PDT treatment, and the relationship between PpIX distribution and PDT treatment efficacy at depths > 1mm is not fully understood. While surface imaging techniques provide useful diagnosis, dosimetry, and efficacy information for superficial tumors, these methods cannot interrogate deeper tumors to provide in situ insight into spatial PpIX distributions. We have developed an ultrasound-guided, white-light-informed, tomographic spectroscopy system for the spatial measurement of subsurface PpIX. Detailed imaging system specifications, methodology, and optical-phantom-based characterization will be presented separately. Here we evaluate preliminary in vivo results using both full tomographic reconstruction and by plotting individual tomographic source-detector pair data against US images.

Keywords: Ultrasound-guided, tomography, spectroscopy, PpIX, subsurface fluorescence

1. INTRODUCTION

An ultrasound-guided, white-light informed, tomographic spectroscopy system is underdevelopment for the quantification of subsurface protoporphyrin IX (PpIX) for diagnostic and treatment evaluation purposes [1, 2]. PpIX is an endogenous sensitizer utilized in photodynamic therapy (PDT) for the treatment of actinic keratosis as well as ‘off-label’ treatment of squamous and basal cell carcinomas (SCC, BCC) [3, 4]. Topical application of aminolevulinic acid (ALA) promotes PpIX production preferentially in pathological conditions of the epithelium such as SCCs and BCCs. Thus ALA-PpIX PDT could provide simple, targeted ablative treatment, if PpIX production could be reliably promoted preferentially in pathological tissues.

This potential for success has been

![Figure 1. Sample source-detector pair sensitivity matrix (arbitrary units). With the 3rd source channel illuminated, this matrix provides the spatial fluorescence origin sensitivity of measurements made using the 3rd detector channel.](image-url)
reached for superficial skin tumors (depth < 1mm), but successful treatment of sub-surface lesions has yet to be achieved [5, 6], likely due to limited availability of ALA [7]. Several techniques to increase ALA penetration are under development [8-11] and proper investigation of the effect of these techniques will require in vivo, subsurface quantification of the PpIX produced [12].

The ultrasound-guided tomographic spectroscopy (UTS) system used here combines fluorescence spectroscopy with diffuse optical tomography to reconstruct fluorophore concentrations in US-defined regions. The tomographic aspect provides sampling from different optical paths within the tissue (Figure 1), which when combined allow for reconstruction of region-specific fluorescence origin based on the sensitivity of each source-detector optical path to a fluorescence in a given region. The reconstruction model, generated and solved using the NIRFAST software package, is based on optical properties determined using white-light spectroscopic fitting. The UTS imaging system, still under development, has the capability to interrogate subsurface light paths, which has been demonstrated in simulations and in tissue optical phantoms. In this manuscript, we present preliminary in vivo application of the UTS system and method for subsurface quantification of PpIX in subcutaneously implanted extra-cellular matrix proteins (Matrigel) in nude mice.

![Tomographic spectroscopy system diagram](image)

**Figure 2.** Tomographic spectroscopy system diagram, showing optical train and sample spectra for excitation-transmission mode, fluorescence mode, and white light mode. Note the five detector channel spectrometers are read simultaneously, while each source channel is illuminated sequentially, for a total of 20 measurements in each mode. The 2 OD filter in excitation-transmission mode is required to reduce signal intensity such that the minimum spectrometer integration time (1ms) does not lead to spectrometer saturation. For white light spectroscopy, the 633nm source laser is shuttered and a separate white light lamp is coupled to an additional fiber at one end of the fiber array.

## 2. MATERIALS AND METHODS

**Imaging System**

Detailed description of the UTS system hardware, functionality, and usage workflow is presented in a separate manuscript (Cite Alisha’s manuscript). The system, diagrammed in Figure 2, is composed of 4 source and 5 detector fibers (600μm custom fibers, LGOptics, Germany), arranged in an alternating line with 2.5mm spacing between fibers. Thus the closest source-detector separation is 2.5mm, and the furthest is 17.5mm. The 4 source fibers are coupled to a 1x4 fiber switch (Piezosystem Jena, Hopedale, MA) for serial illumination with the excitation source during excitation-transmission measurement and fluorescence spectroscopy (Figure 3). The excitation source is a 633 nm laser system with current and temperature control (Model 7404, Intense Co., North Brunswick, NJ). The excitation light passes through a filter wheel before being coupled to the 1x4 fiber switch. The filter wheel contains a 2-OD neutral density filter (NE20B, Thorlabs, Newton, New Jersey, USA), a beam stop, and a 650nm short pass filter (FES0650, Thorlabs).
On the detection side, each of the 5 detection fibers passes through a moveable filter block (MFF001, Thorlabs) with a 655nm long pass filter (655ELPF, Omega Optics, Austin, Texas, USA) and terminates at a spectrometer (USB2000+, Ocean Optics, Dunedin, Florida, USA). The moveable filters are modified with custom limit switch mounts for position feedback, and are controlled via data acquisition cards (DAQs, NI USB 6009, National Instruments, Austin, Texas, USA) using LabView virtual instrument software (LabView v8.4.2, National Instruments). The filter wheel, spectrometers, and 1x4 fiber switch are controlled via USB using LabView. The spectrometers for all 5 detection channels can be read in parallel. Four source channels and five detection channels provide a total of 20 source-detector pair measurements in each imaging mode.

The imaging protocol involves three steps. First, the transmitted excitation signal is acquired with the 633nm excitation light passing through the 2OD filter and each of the 4 source channels sequentially. This signal will provide normalization for variations in source intensity between channels. The 650nm moveable long pass filters are disengaged for transmitted excitation measurement. Second, for fluorescence spectroscopy, the 633nm excitation source passes through the 650nm short pass filter in the filter wheel to remove any contaminating signals. On the detection side, the moveable 655nm long pass filters are engaged to remove the excitation signal. Again, 5 measurements are captured for each source channel, for a total of 20 spectra. Finally, white-light spectra are acquired to inform reconstruction model optical properties. A white light source (HL-2000-FHSA, Ocean Optics) was coupled to an additional 600μm custom fiber placed in line with the other 9 fibers. With the 633nm excitation source shuttered, white light spectra were captured at each of the 5 detectors with the white light source fiber at one end of the fiber line. In all three imaging modes, spectrometer integration times are automatically adjusted for maximum signal without saturation. The entire imaging process takes approximately 60s.

Prior to tomographic spectroscopy (TS) imaging, the region of interest is found and imaged using US. US imaging was performed using a Sonix Tablet (Ultrasonix, Richmond, BC, Canada) high-frequency ultrasound system with a 40 MHz transducer (L40 8/12, Ultrasonix). The two imaging modalities are co-registered via a sliding v-block that allows the US and TS transducers can be manually interchanged while maintaining the same imaging position.

**Processing**

US images were manually segmented into two regions, inclusion and background, using Matlab, based on the hypoechoic nature of Matrigel injections relative to background tissues. The segmented image was used to construct a region mask, which was extended laterally to cover the full TS imaging field, which is 10mm wider than the US imaging field. The region mask was then input into the finite-element meshing algorithm, which takes into account the non-uniform height of the fiber optics sources and detectors due to an uneven imaging field.

![Figure 3. Experimental tomographic spectroscopy setup for imaging mouse hind flank. A) Position of TS fiber optics transducer, with source channel 1 illuminated. The imaging setup is normally covered by black fabric to block contaminating room light, but the shroud is removed for imaging purposes. B-E) Serial illumination of the 4 source channel fibers.](image)

![Figure 4. Spectral plot showing a representative spectral fit. The measured data is fit using a combination of autofluorescence, PpIX, and PpIX 675nm photoproduction bases.](image)
White-light spectra were spectrally fit for tissue optical properties, including absorption and scattering, using a diffusion theory algorithm [15]. The broadband spectra, 550-900 nm, were first calibrated using measured intensity and model estimates for a known 1% Intralipid (Fresenius-Kabi, Bad Homburg, Germany) phantom. Measured experimental data, with intensity units of counts/s, was converted into units of photons/mm$^2$ by multiplying by $I_{\text{mode}}/I_{\text{measured}}$ of the Intralipid calibration phantom. The calibrated data was then used to estimate tissue chromophore concentrations, including oxy- and deoxygenated hemoglobin and water. Collagen/melanin scattering and absorption was also considered. The estimates were used to calculate bulk tissue absorption and scattering properties at 633 nm and 700 nm, the respective PpIX excitation and emission wavelengths used in this study.

Transmitted excitation spectra and fluorescence spectra were time-normalized, spike-filtered using a 3-point median filter, then smoothed using a 14-pt (~5 nm) moving average filter. Fluorescence spectra was spectrally fit using basis spectra for tissue/system autofluorescence, PpIX emission, and PpIX photoproduct emission using a linear-least-squares fitting algorithm over the range of 660-800 nm (Figure 4). Tissue/system autofluorescence spectra used were source-detector pair specific spectra imaged in pre-injection mice. PpIX and PpIX 675 nm photoproduct spectra were obtained from the Oregon Medical Laser Center library (omlc.ogi.edu). PpIX fluorescence signal was taken as the fit value obtained using normalized basis spectra. Excitation signal was taken as the 633 nm value of the transmitted excitation spectra.

Prior to fluorophore reconstruction, the individual, Born-normalized, source-detector values of fluorescence were plotted against the position they interrogated on segmented mask. The PpIX signal in the inclusion relative to the background was evaluated qualitatively to ascertain if direct observation of Born-normalized fluorescence values is sufficient to inform spatial distribution of PpIX.

White-light determined tissue optical properties were added to the masked finite-element mesh. The white-light informed optical mesh was used to model the propagation of transmitted excitation light using diffusion theory in the NIRFAST software package [16] in Matlab. Fluorescence signals were calibrated using a ratio of measured and modeled excitation light propagation, in a process similar to Born normalization. An iterative algorithm was used to estimate the fluorophore concentration, minimizing the sum-of-squares difference between calibrated and modeled source-detector measurements, assuming uniform distribution. This value was divided by 10 and used as a starting guess for fluorophore concentration in each mesh region. The mesh was then used to iteratively estimate the fluorophore concentration in each region. The quantum efficiency of the fluorophore was estimated to be ~0.0005 based on previous work and the value that best fit the observed data.

Animal Tumor Models
To create in vivo models of high stromal content skin tumors, 150 μL extra-cellular matrix protein (Matrigel, BD Biosciences, San Jose, California, USA) was injected subcutaneously over the hind leg muscle in nude mice. Matrigel forms a solid gelatinous network rapidly at physiological temperatures; the injected Matrigel was not imaged until 1 h after injection at which point it had solidified completely. PpIX (P8293, Sigma-Aldrich) was dissolved in DMSO at 1 mg/mL and then serially diluted to achieve final concentration 0.01 mg/mL. To simulate ALA-induced PpIX in this Matrigel inclusion, PpIX-DMSO solution was added to the Matrigel to reach final concentration of 0.1 μg/mL prior to injection. PpIX was protected from light during all steps to minimize photobleaching and generation of photoproducts. For imaging purposes, mice were anesthetized via isofluorane/oxygen flow.

3. RESULTS

Animal Models
Matrigel injections accurately simulated sub-cutaneous tumors in both stiffness and ultrasound response. In each case the Matrigel inclusion formed a solid sub-cutaneous mass with 1 h. Injections were directly discernable by palpation and during US imaging by the hypo-echoic nature of Matrigel-water inclusions compared to surrounding tissues. Inclusions were successfully imaged using the dual-mode, co-registered transducer v-block (Figure 3), though some inclusions presented difficulty during transition from US imaging to TS imaging due to sliding.
Image processing

Spectral data was time-normalized and smoothed as described. Spectral fitting for PpIX signal typically yielded expected results, with consistent autofluorescence levels and PpIX photoproduct levels remaining a fraction of the PpIX emission signal (Figure 4). In some cases the least-squares spectral fit yielded no photoproduct and/or no PpIX signal. For longer source-detector separation distances of 12.5mm and 17.5mm, the signal-to-noise ratio was lower and the spectral fits were expectedly worse. A fitting window of 660-800nm was determined to provide the most accurate spectral fits. A representative plot of source-detector values for excitation transmission, fluorescence, and transmission-normalized fluorescence is provided in Figure 5. Note excitation-normalized fluorescence increases with distance from the source when imaging a fluorophore because more fluorescence is accumulated.

White light spectra were fitted for tissue chromophores including oxy- and deoxy-hemoglobin and water. The results were consistent with expected values and previous work. These fits were used to calculate the bulk tissue optical properties at PpIX excitation and emission wavelengths. These optical properties were then used to construct an accurate optical finite element model for reconstruction.

US images were readily segmented based on the consistent contrast between Matrigel inclusions and background tissue (Figure 6A). These images were segmented in Matlab by

Figure 5. Excitation-transmission, fluorescence, and normalized fluorescence values for all source-detector pairs. Red lines represent the current source channels and dotted vertical lines separate sequential source channels (i.e. in A, far left, source 1, located between detector 1 and 2 is illuminated). A) Excitation-transmission values. These values represent the spectral component at the excitation wavelength, 633nm. Note the signal drops off with distance from the source, as expected. B) Fluorescence values. These values represent the fitted PpIX values from spectral fitting. Again note that the signal drops off with distance from the source as expected. C) The transmission-normalized fluorescence values. These values represent fluorescence/transmission values for each source-detector pair. Note here that the Born-normalized signal is expected to increase with distance from the source, because more fluorescence emission is accumulated over longer optical paths.

Figure 6. Raw data approach to evaluating tomographic fluorescence signals using nearest-neighbor source-detector pairs. A) Original ultrasound image. B) The 2-region mask supplied to the meshing algorithm, with sources and detectors labeled. C) Born-normalized (Fluorescence/Excitation-Transmission) signals for nearest-neighbor source-detector pairs (arbitrary units). The positions correspond to the tissue area interrogated by the source-detector pair. For example, the source-1–detector-1 pair interrogates a space roughly centered at a position between the two fibers, at sx = 1.25mm.
manually defining a polygon mask (Figure 6B). The Born-normalized PpIX fluorescence signal for nearest-neighbor source-detector pairs was then plotted against position along the segmented image and the spatial distribution of fluorescence signal agreed with the US-determined position of the inclusion (Figure 6C). The contrast-to-background ration determined by this method is ~8. However, without knowing the absolute concentrations of PpIX in the background, the actual concentration in the inclusion cannot be determined.

The segmented image was used to generate an optical finite element mesh as shown in Figure 7A. The spectrally fit fluorescence values, along with the excitation-transmission values, were input into NIRFAST along with the mesh for reconstruction of regional fluorophore concentrations (Figure 7B). In this case the contrast-to-background ratio was calculated as 245. The actual concentration in the inclusion can be reconstructed if we assume a quantum efficiency for PpIX of ~0.0005.

5. DISCUSSION

Regional PpIX measurement

The preliminary results presented here demonstrate the ability of the USTS system and methodology to measure subsurface PpIX. USTS imaging provides qualitative spatial distribution information while tomographic reconstruction provides quantitative inclusion-to-background information. Absolute PpIX concentrations cannot be calculated without knowledge of the PpIX quantum efficiency. Fluorophore quantum efficiency is not easily measure directly, and varies significantly depending on the solvent and environment. The results suggest that a quantum efficiency calibration value can be determined which allows calculation of absolute PpIX concentrations in vivo.

Limitations

The results presented here are preliminary and only include a few data points. More animals and PpIX concentrations must be imaged before full conclusions can be made. The biggest limitation in the current method is the mobility of the injected Matrigel inclusions. While the Matrigel does form a solid mass when injected subcutaneously, this mass does not typically adhere to the underlying muscle tissue, and thus can move considerably during imaging. This mobility presents problems for the co-registered, dual-mode imaging methodology used here because the dual-transducer sliding v-block requires withdrawal of the US transducer to place the TS transducer, and during US transducer movement, the sticky US gel adheres to the mouse skin and moves the Matrigel inclusion. Furthermore, when the TS transducer is placed, the sliding motion of the optical fibers as they contact the inclusion can also displace the inclusion slightly. While this mobility issue will not be as significant when imaging actual tumors, for they typically attach to the surrounding tissues, the issue does present problems for small animal imaging, and an optically compatible stabilizer ring is under development to reduce inclusion mobility in future imaging.

Future work

Beyond extending this study to include a full range of PpIX concentrations, the system and methodology presented here will be applied to imaging ALA-induced PpIX in healthy skin and animal tumor models. Once the system and methodology have been fully validated for sub-surface quantification of PpIX, work will begin to adapt the USTS system and methodology to the clinic for skin tumor diagnosis and PDT efficacy evaluation.

Figure 7. Finite element mesh and diffuse optical tomography reconstruction results. A) FEM optical mesh with sources (o) and detectors (x) labeled. The sources appear to be inside the sample because they are positioned 1/(optical scattering parameter) inside the mesh using method of images to avoid interface instabilities during diffusion modeling and reconstruction. B) 2-Region reconstruction results (arbitrary units) with detectors (x) labeled. In this case, the contrast-to-background ratio, 245, is somewhat meaningless because PpIX was injected into the region of interest and there is relatively no PpIX in the surrounding tissues in the mouse. Without knowledge of the background value or fluorophore quantum efficiency in vivo, the absolute PpIX concentration cannot be determined.
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7. REFERENCES