In vivo validation of high frequency ultrasound-guided fluorescence tomography system to improve delivery of photodynamic therapy

Akshat Paliwal\textsuperscript{a}, Sason Torosean\textsuperscript{d}, Josiah Gruber\textsuperscript{d}, Julia O’Harad\textsuperscript{d}, Tayyaba Hasan\textsuperscript{e}, Brian Pogue\textsuperscript{d}, Edward V. Maytin\textsuperscript{a,b,c}

\textsuperscript{a} Cleveland Clinic Lerner College of Medicine, \textsuperscript{b}Dept. of Dermatology and \textsuperscript{c}Dept. of Biomedical Engineering, Lerner Research Institute; Cleveland Clinic, 9500 Euclid Ave., Cleveland, OH 44195, U.S.A;
\textsuperscript{d}Thayer School of Engineering, Dartmouth College, Hanover, NH 03755, U.S.A.
\textsuperscript{e}Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard, Boston, MA 02114

ABSTRACT

Photodynamic therapy (PDT) for skin cancer is sometimes only partially effective, due to inadequate levels of the fluorescent drug (photosensitizer, PS) and due to heterogeneous distribution of PS within the tissue. To image the PS distribution within skin tumors, we have developed a fluorescence tomography system (FTS) that combines a fluorescence detection array with a high frequency ultrasound (HFUS) transducer. In this paper we describe in vitro and in vivo validation of this new system. The target fluorophore for detection was Protoporphyrin IX (PPIX). Validation experiments were performed in vivo using a subcutaneous tumor model in which A431 tumor-bearing mice were treated with 5-aminolevulinic acid to induce production of PPIX. FTS reconstructions were compared with standard histology and with data from bulk tumor slices imaged ex vivo on a fluorescence scanner. Reconstructed images obtained from the FTS were correlated with the histology and the ex vivo scans, confirming several-fold increases in PPIX fluorescence in the skin and in the tumor relative to the surrounding tissues. Our data demonstrate the feasibility of using the FTS for subsurface imaging of PPIX in skin carcinoma in vivo. Future aims are to use this device for individualized treatment planning, in order to improve overall patient responses to PDT.

Keywords: fluorescence tomography, high frequency ultrasound, photodynamic therapy, protoporphyrin IX, multimodal imaging

1. INTRODUCTION

1.1. Nonmelanoma skin cancers and Photodynamic therapy (PDT)

The incidence of skin cancer has been increasing 3-8% every year since the 1960s, and it is the most commonly occurring cancer in the white populations of Canada, United States and Australia.\textsuperscript{1} Non-melanoma skin cancers, such as basal cell carcinoma and squamous cell carcinoma comprise the majority of these malignancies.\textsuperscript{2,3} The standard therapy for these types of cancers has been either simple excision or MOHS micrographic surgery.\textsuperscript{4} Unfortunately, these procedures lead to scarring and carry a risk of post-procedural infection.\textsuperscript{5} Sometimes, when patients have lesions in sensitive areas such as the face or have a large number of lesions distributed over a broad area, surgery and surgical scarring can become problematic.\textsuperscript{4} The use of photodynamic therapy (PDT) to treat pre-cancerous lesions called actinic keratosis (AKs) has been approved for use in many countries including the USA, and PDT is starting to be used routinely in some academic centers to treat recurrent squamous cell carcinoma.\textsuperscript{5}

PDT uses a molecule called the photosensitizer (PS) in combination with light to selectively target cancer cells. In PDT, the interaction between the photosensitizing agent, light, and oxygen can be best described as a two-step process. In the first step, PS is administered to the patient (either intravenously, or topically in the case of a superficial skin tumor) and is allowed to be taken up by the target cancer cells. In the second step, the PS is activated in the presence of oxygen by a
specific wavelength of light directed toward the target tissue. Because the PS is preferentially absorbed by hyperproliferative tissue, and the light source is directly targeted on the lesion, PDT provides dual selectivity that minimizes the damage to non-tumor tissue. In cutaneous oncology, aminolevulinic acid (ALA) is commonly used as a precursor drug; ALA is the first compound in the porphyrin synthesis pathway that leads to hemoglobin in mammals. Within cancer cells, porphyrins (specifically protoporphyrin IX, or PPIX) are selectively synthesized at higher amounts than in normal cells. Once activated with light, the PPIX can either emit fluorescent light (which is detectable either visually or using a fluorimeter), or can emit reactive oxygen species such as singlet oxygen (leading to cell death). In both laboratory and clinical studies, after topical application of ALA, cancer cells accumulate more PPIX than non-cancer cells and this phenomenon has led to the use of ALA and PDT for the treatment of AKs and Bowen's disease (squamous cell carcinoma in situ).

The advantages of PDT over surgery and cryotherapy are that PDT causes minimal tissue disruption, is completely non-invasive, and has superior cosmetic outcome in sensitive areas like the face and the scalp. Moreover, beyond direct phototoxic effects on target tissue, PDT generally has a low potential for causing DNA damage, mutations, and carcinogenesis.

1.2. Imaging subcutaneous and intradermal skin tumors

PDT as currently practiced is not always fully effective, whether due to inadequate levels of photosensitizer or due to a heterogeneous distribution of the PS within the tumor tissue. The effectiveness of PDT for non-melanoma skin cancers can be improved with agents that increase uptake and penetration of the photosensitizer into the skin or agents used in combination with ALA that synergistically up-regulate the selective production of PPIX in cancer cells. The production of PPIX in skin tumors is a dynamic process, and methods to measure and study the distribution of PPIX in vivo within a tumor in real time do not exist. Designing a study to measure and investigate the distribution of PPIX in tumors requires a large number of animals, and this number increases if one adds additional compounds to act synergistically with ALA-PDT to optimize clinical responses for deep and/or resistant tumors. Studies to optimize ALA-PDT for skin cancer in humans is even harder, considering the heterogeneity in skin types, morphology of the tumors, and fact that a tumor can only be biopsied at a static point in time. Therefore, we have developed a fluorescence tomography system (FTS) for imaging of skin tumors to study the dynamic changes in concentration of PPIX and its distribution within tumors. While PDT can be used for many different cancers, we are concentrating here upon creating a device specific for subsurface imaging of skin cancer (primarily basal cell and squamous cell carcinoma).

This device combines high frequency ultrasound with an array of fibers for optical imaging. The HFUS shows us the ultrastructure of dermis, epidermis, hair follicles, and tumor, while an overlay of fluorescence signatures from the fiberoptic array gives us the distribution of PPIX within these structures. Such information could tell us whether sufficient levels of PPIX have been achieved within the tumor, and whether the distribution of PPIX is uniform or heterogeneous. It can also help us study the magnitude and depth of PPIX upregulation while using compounds that act synergistically with ALA.

1.3. Previous studies of in vivo PPIX measurement

Zhu et al. have been able to couple fluorescence detection array and near infrared tomography with ultrasound for breast tissue imaging, and other groups have explored optical coherence tomography (OCT) and HFUS for skin tumor imaging. The notion of combining a fluorescence detection array with a 2-D structural image has been conceptually validated. For the last few years, a device called the Aurora imaging system has been used to take in vivo measurements of PPIX in skin tumors. The primary limitations of this device have been that it has a relatively shallow depth of penetration and it provides no data on the distribution of PPIX. Modalities such as OCT offer excellent image resolution, but can only image shallow cutaneous tumors (max depth ~0.5 mm). The FTS described in this paper is a
more robust system which can image both cutaneous and subcutaneous tumors (max depth ~1 cm) and provides information on both relative concentration of PPIX and its distribution.

2. METHODS

2.1 Instrumentation of the FTS
High frequency ultrasound provided the ideal depth for imaging subcutaneous squamous cell carcinoma tumors. An off-the-shelf Visual Sonics HFUS system was used with a 40 MHz Visual Sonics Vevo 704 probe. The fluorescence detection array that couples to the Visual Sonics probe was designed based on the field of view and width of the 40 MHz HFUS probe. It was determined that 4 fiberoptic laser sources with 5 detectors would fit on the array allowing for the proper width of the fibers and proper spacing for fiber protection. We sought to optimize functionality of the hardware while keeping commercial availability, cost and modularity in mind.

Figure 1: Schematic of FTS shows the VisualSonics HFUS system. INSET: Configuration of the fluorescence tomography array with alternating source and detector fibers.
The schematic of the FTS is shown in Figure 1. includes a 635 nm wavelength laser source controlled by LabView data acquisition (DAQ) card. The five detection fibers measure 200 µm are attached to individual spectrometers (Ocean Optics). These spectrometers have a bandwidth from 540 to 1210 nm. The system utilizes a National Instruments USB data acquisition card to deliver the raw fluorescence spectrum data to our analysis software, programmed in LabView. The operator starts by placing some ultrasound gel on the gelatin phantom, or the anesthesized mouse. After starting the system, the Labview program cycles through the four laser sources one at a time. Fluorescence data spectrum is recorded at each detector while one of the lasers is tuned on. With activation of each source fiber, five data sets are obtained from the five detectors. Once the system cycles through all four lasers, a total of twenty spectral data sets has been collected.

A gelatin phantom or a mouse with a subcutaneous tumor is placed on a calibrated micro-manipulator stage allowing for precisely measurement of tumor movement during data collection. HFUS imaging is used first to obtain a suitable plane for FTS imaging that includes the greatest cross-sectional area of the tumor. Then, a cross-sectional image of the tumor is taken using the HFUS probe. The operator then shifts the FTS by 7 mm before taking optical measurements on the same plane of the sample. This is necessary to accommodate for a 7 mm offset between the center of HFUS probe and the florescence array.

As seen in Fig. 2, fluorescence data acquisition and HFUS image acquisition occur independently. The optical data undergoes data calibration in LabView, while the HFUS image is segmented and a mesh is generated using MatLab, to divide the image into different areas that are determined by the acoustic properties of the tissue or phantom. Then, a program written in Matlab, called NIRFAST, is used to reconstruct a gradient image of PPIX fluorescence.

2.2 Validation using Gelatin phantoms

Tissue phantoms were utilized to validate the FTS and to simulate properties of skin and tumor.

Homogeneous liquid tissue phantoms were made in phosphate buffered saline by adding 1% Intralipid for scattering and 2% India ink for absorption. Various concentrations of PPIX were added to the phantoms, depending upon the specific experiment; 5% Tween-20 was added to the liquid to monomerize the PPIX.

To create gelatin phantoms, 0.5-1% agarose was mixed with 99-99.5% water and heated in a microwave for 20 seconds to boiling temperature. Next, the liquid agarose was added to the liquid phantom solution (described above) and allowed to cool in 4 °C in 51 mm diameter Petri dishes for 15 minutes. Two- and three-layer phantoms were created by cutting thin slices of the gelatin using a razor blade and layering it on top or below gelatin phantoms with different PPIX concentration. Both the homogenous liquid and gelatin phantoms with known concentrations of PPIX were used to calibrate the system for in vivo imaging.
2.3 In vivo imaging in nude mice using A431 skin carcinoma model

A431 cells from a human SCC cell line were used in a subcutaneous tumor model in nude mice. These mice were placed on a chlorophyll free diet, and $3 \times 10^6$ A431 cells in combination with Matrigel were injected at two sites subcutaneously in immunocompromised nude mice, leading to the development of palpable, subcutaneous tumor nodules in ~7 days. All mice received ALA by 0.5 ml of intramuscular 75 mg/kg dose. The mice were anesthetized with isoflurane, and then imaged at 3 h and 24 h time points. The animals were sacrificed immediately after completion of FTS imaging, and the tumors harvested for ex vivo imaging. The extracted tumors were bisected using a razor blade and one half imaged using confocal imaging, while the other half were imaged using a Typhoon fluorescence scanner. The samples imaged on the Typhoon scanner were then sent for H&E staining.

3. RESULTS

3.1 HFUS imaging and segmentation of heterogeneous tumors

Figure 3: A431 skin carcinoma tumors in nude mice of various appearances (a-c) were imaged with the HFUS system. These images were then segmented using LabView (d-f).

The examples in Figure 3 show the following. Tumor (a) is hypoechoic with surrounding hyperechoic tissue. Tumor (b) is hyperechoic surrounded by hypoechoic tissue but has very sharp margins. Tumor (c) is hyperechoic in hyperechoic background tissue, and displays relatively poor contrast with its surroundings. Successfully segmenting these images of tumors with different properties is the first step in 2D reconstruction of the PPIX fluorescence gradients.
3.2 *Ex vivo* imaging using H&E histology, typhoon and confocal microscopy

![Image of A431 skin carcinoma tumor excised from nude mouse stained with H&E and imaged in vivo using HFUS.](image)

The example is Figure 4 compares the histological appearance of a subcutaneous multi-nodular A431 skin carcinoma tumor in a nude mouse with its appearance on in vivo HFUS imaging. Several of these comparisons were made to determine that 40 MHz HFUS was a good method to image these tumors with sufficient resolution.

![Image of A431 skin carcinoma tumors excised from mice 3 hours after ALA injection and 24 hours after ALA injection, imaged using confocal microscopy with filters set for imaging PPIX.](image)

The excised subcutaneous A431 tumors shown in Fig. 5 were imaged *ex vivo* using a Typhoon fluorescence scanner with setting to selectively image PPIX fluorescence. In panel (a) tumors were excised 3 hours after ALA injection and in panel (b) the tumors were excised 24 hours after ALA injection. The bright band on in the tumor images is mouse skin that...
overlies the tumor. It can be seen that the skin is consistently producing much more PPIX than the tumor at both the 3h and 24h time points. Comparing the images from 3 h and 24 h time points shows that both the mouse ski and the subcutaneous tumor produce more PPIX at the 3h time point. A subcutaneous A431 tumor imaged using confocal microscopy 3h post ALA (c) treatment confirms the results seen with Typhoon imaging. The skin in the bottom right corner of the confocal image is much brighter than the tumor nodule shown in (c) and magnified in (d).

3.3 Image Reconstruction using FTS

![Image](image-url)

**Figure 6:** In vivo imaging of A431 skin carcinoma tumor in nude mouse. A reconstructed image developed using the FTS is presented (a) and it shows a relatively low concentration of PPIX in the tumor surrounded by high concentration of PPIX in skin and surrounding tissue. This pattern is reconfirmed using ex vivo imaging.

The FTS reconstructed image in Figure 6 shows the skin overlying the tumor is more fluorescent than the A431 tumor. This result is consistent with the skin to tumor fluorescence gradient seen in ex vivo imaging.

4. CONCLUSIONS

In this FTS design, we have validated that high-frequency ultrasound provides a clear anatomic image with sufficient resolution to visualize skin tumor boundaries. The segmenting software is able to take images in different planes, tumor and background tissues with different echogenicities and segments them appropriately. From our ex vivo imaging, we have determined that our tumor model of subcutaneous A431 squamous carcinoma tumors in nude mice shows that these tumors have increased fluorescence at 3h post ALA treatment compared to 24h post ALA treatment. We have also determined that the skin is significantly more fluorescent than the tumor at both the 3h and 24h time points. Both these findings have been confirmed using the FTS reconstructions. In the future, we plan to quantify the in vivo fluorescence measurements and would like to compare the fluorescence at different depths within the tumor. We are also continuing to do simulations and phantom studies to test the robustness of the system while varying the fluorescence in different layers of tissues, varying the distance of the probe from the tissue, varying the angle of the probe, etc. We are also using...
these phantom studies to improve the signal-to-noise ratio of the system. We hope that the FTS will be used to study the role of Vitamin D and various other molecules in enhancing the efficacy of PDT. Ultimate goal would be to seek IRB approval for using the FTS on human subjects undergoing PDT therapy at the Cleveland Clinic and evaluating the system’s performance.

5. ACKNOWLEDGMENTS

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6. REFERENCES