

Quantitative imaging of scattering changes associated with epithelial proliferation, necrosis, and fibrosis in tumors using microsampling reflectance spectroscopy

Venkataramanan Krishnaswamy

Dartmouth College
Thayer School of Engineering
8000 Cummings Hall
Hanover, New Hampshire 03755

P. Jack Hoopes

Dartmouth Medical School
Departments of Surgery and Medicine
Borwell Research Building
Lebanon, New Hampshire 03756
and
Dartmouth College
Thayer School of Engineering
8000 Cummings Hall
Hanover, New Hampshire 03755

Kimberley S. Samkoe

Dartmouth College
Thayer School of Engineering
8000 Cummings Hall
Hanover, New Hampshire 03755

Julia A. O'Hara

Dartmouth College
Thayer School of Engineering
8000 Cummings Hall
Hanover, New Hampshire 03755
and
Dartmouth Medical School
Department of Radiology
Borwell Research Building
Lebanon, New Hampshire 03756

Tayyaba Hasan

Massachusetts General Hospital
Wellman Center for Photomedicine
40 Blossom Street
Boston, Massachusetts 02114
and
Harvard Medical School
Department of Dermatology
55 Fruit Street
Boston, Massachusetts 02115

Brian W. Pogue

Dartmouth College
Thayer School of Engineering
8000 Cummings Hall
Hanover, New Hampshire 03755
and
Dartmouth Medical School
Department of Surgery
Borwell Research Building
Lebanon, New Hampshire 03756
and
Massachusetts General Hospital
Wellman Center for Photomedicine
40 Blossom Street
Boston, Massachusetts 02114

Abstract. Highly localized reflectance measurements can be used to directly quantify scatter changes in tissues. We present a microsampling approach that is used to raster scan tumors to extract parameters believed to be related to the tissue ultrastructure. A confocal reflectance imager was developed to examine scatter changes across pathologically distinct regions within tumor tissues. Tissue sections from two murine tumors, AsPC-1 pancreas tumor and the Mat-LyLu Dunning prostate tumor, were imaged. After imaging, histopathology-guided region-of-interest studies of the images allowed analysis of the variations in scattering resulting from differences in tissue ultrastructure. On average, the median scatter power of tumor cells with high proliferation index (HPI) was about 26% less compared to tumor cells with low proliferation index (LPI). Necrosis exhibited the lowest scatter power signature across all the tissue types considered, with about 55% lower median scatter power than LPI tumor cells. Additionally, the level and maturity of the tumor's fibroplastic response was found to influence the scatter signal. This approach to scatter visualization of tissue ultrastructure *in situ* could provide a unique tool for guiding surgical resection, but this kind of interpretation into what the signal means relative to the pathology is required before proceeding to clinical studies. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3065540]

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1 Introduction

One of the significant challenges during surgery is the lack of obvious contrast between normal and tumor tissues in the margins of many invasive tumors. While surgical microscopes can be fitted with color filtering,^{1,2} polarizers,^{3,4} or spectro-

Address all correspondence to Venkataramanan Krishnaswamy, Thayer School of Engineering, Dartmouth College, 8000 Cummings Hall-Hanover, NH 03755. Tel: 603-646-2393; Fax: 603-646-3856; E-mail: venkat@dartmouth.edu

scopic channels,⁵ there is still room for the definition of tools that would provide better delineation of the tissue ultrastructure *in situ* and that can readily gain clinical adoption. One key area of work is in the idea of decoupling the signal scatter from absorption *in situ*, which would be extremely beneficial, since the origin of absorption signals is thought to be completely separate from the features causing scatter.⁶⁻⁹ Several techniques exist for imaging features related to scatter using reflectance polarization,^{3,10-13} yet comparatively less attention has been paid to imaging the scatter spectrum directly. There have been some unique designs for novel optical fiber-based probes,¹⁴⁻¹⁷ which have the potential to guide biopsy or provide differential information about tissue status, and extending the signal processing of these devices into an imaging mode would be quite beneficial. This paper looks at one approach to broadband spectral reflectance imaging to quantify scatter in an imaging geometry, where the field of view is large enough to allow eventual translation as a surgical pathology aid. In addition, the approach allows easy quantification of the scattering coefficient heterogeneity within tissue samples; this information is critical to treatment planning in optical treatment such as photodynamic therapy.¹⁸⁻²⁰

Scatter analysis of cells and tissues has provided some fundamentally exciting potential in recent years. Angle-resolved or coherence-based reflectance methods have been shown to allow quantification of the scatter origin features in tissue,^{17,21-25} which can be robust measures of pathologic change. The shape and size of scattering features can produce resonances in the angularly resolved emission or in the wavelength-resolved emission, which could be extracted from the overall spectral data. The techniques that are based on locally resolved reflectance measurements are well studied but have not been extended into imaging systems. Since these methods have been shown to work for quantifying scatter and absorption changes in tissue if the target optical properties are within a typical range observed in tissue, they are ideal for extension to imaging, if a reasonable way can be found to measure scatter in small regions such as 100 micron diameter, which is equivalent to approximately one mean scattering length in tissue.²⁶⁻³⁰ The logical approach to this is to develop a raster-scanning system that takes full broadband wavelength data as reflected from the tissue surface. The optical design constraints on this system are many, in that with broadband light, the optics have to be chromatically corrected and work quickly enough to allow capture of the broadband data at every pixel.

A new raster-scanning reflectance imager has been designed here, based on these techniques, to directly quantify tissue scatter changes *in situ*. The system is outlined and the features of the detected scatter signals are analyzed with two tumor models, looking at the variation in scattering parameters within the tumor line. The system has good potential for extension to clinical imaging, but the heterogeneity observed in real tumor tissue can be enormous, and so this study focuses on classifying the signal types obtainable from localized scatter and directly comparing them to what is seen by a pathologist. The ability to use this data for diagnostic studies and treatment planning is discussed.

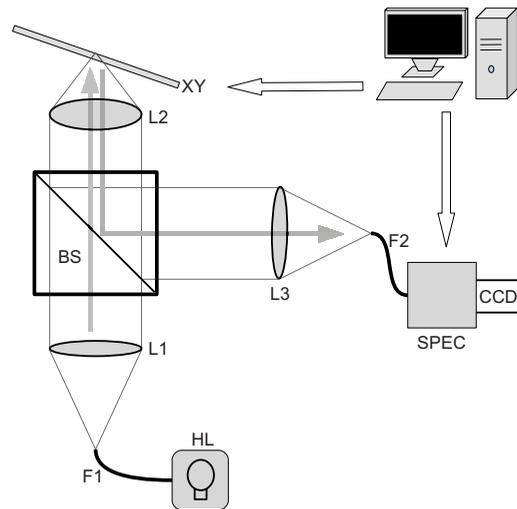


Fig. 1 Schematic of the raster-scanning scatter imager. The solid semi-transparent arrows show the illumination and detection beam paths. Identical achromatic lenses (L1 and L3), the objective lens (L2), the beamsplitter (BS), the illumination fiber (F1), and the detection fiber (F2) are all arranged in the standard confocal geometry. The distal end of the detection fiber is coupled to a CCD-based imaging spectrometer (SPEC), which records the remitted irradiance spectrum.

2 Materials and Methods

2.1 Scatter Imaging System

The scatter imaging system consists primarily of a confocal spectroscopic system having 100- μm illumination spot size and a raster-scanning sample platform built using linear translation stages. The optical and electromechanical subsystems are integrated via a custom-developed LabVIEW (National Instruments, Austin, Texas) interface. Figure 1 shows the schematic of this system, which resembles a traditional confocal microscope but is designed to directly image scatter from tissue. This is achieved by ensuring that the illumination and detection spot sizes on the tissue surface are less than one scattering length (typically 100 μm for tissue)²⁷ over the entire waveband of interest. In this regime, the incident photons are predominantly singly scattered and the detected signal is largely independent of local absorption.

The illumination optics train consisted of a 50- μm core fiber (F1) coupled to a 20-W tungsten-halogen white light source (HL-2000-HP, Ocean Optics, Inc., Dunedin, Florida) placed at the front focal point of an achromatic lens (L1) (PAC040, Newport Corporation, Irvine, California). A 10 \times , 0.28 NA, long working distance, air immersion, plan-apochromatic objective (L2) was used to refocus the light on to the sample plane. The illumination optics train was modeled in ZEMAX optical design software (version July 22, 2004, ZEMAX Development Corporation, Bellevue, Washington) to make sure that the illumination spot size was less than 100 μm over the entire wavelength band used. The 50- μm -diam extended source (fiber core) was modeled as a collection of point sources placed at the extremities of the source field positions. The transverse magnification of the illumination optics train was approximately 0.4. The theoretical spot size at the sample plane was found to be less than approximately 40 μm over a broad wavelength range of

450 nm to 800 nm, with chromatic aberration dominating the point spread function. The theoretical spot size was within 100 μm for a depth of approximately 130 μm on either side of the best focus plane. It should be noted that the microscope objective (L2) lens system was modeled as a single paraxial lens with equivalent specifications, as the actual lens data for the objective was not available. However, the objective is well corrected for common optical aberrations and was expected to deliver a diffraction-limited performance over the design waveband. The spot size was also experimentally measured using the standard knife edge technique and was found to be approximately 89 μm .

The detection optics train used the same microscope objective to pick up the backscattered light from the sample, and a 50/50 beam splitter (BS) was used to separate the illumination and detection beam paths. Another achromatic lens (L3) was used to focus the detected photons onto the proximal face of a 100- μm -core detection optical fiber (F2), which also acted as the confocal pinhole. The size of the detection spot on the target was controlled by the detection fiber's core diameter and the lateral magnification of the optical system. The detection optical train provided a magnification of approximately 2.5, and thus the 100- μm detection fiber picked up light from a 40- μm region overlapping the illumination spot on the sample plane. The distal end of the detection fiber is coupled to an imaging spectrometer (SpectraPro 2156, Princeton Instruments, Inc., Trenton, New Jersey) with a 150 grooves/mm, 500-nm blaze wavelength, ruled grating and a 16-bit, thermo-electrically cooled Cascade 512F EM-CCD (Princeton Instruments, Inc.). The CCD has a field of 512×512 pixels with a pixel size of 16 microns. The spectrometer was calibrated using characteristic spectral lines from a calibrated fluorescent lamp PLS 11W/827 (Philips Lighting, Somerset, New Jersey)³¹ to operate in the wavelength range of 510 nm to 785 nm that encompasses the strong hemoglobin absorption peaks. The spectral resolution was approximately 1 nm.

A custom sample mount was designed to hold removable microscope slides and standard 96-well plates for tissue and liquid phantom measurements, respectively. The mount was integrated into an XY linear translation platform and aligned such that the top surface of the microscope slide lies exactly on the focal plane of the objective lens (L2). The entire optical head was mounted on a vertical translation stage to enable the scan spot to move in and out of the sample plane to allow for precise vertical alignment.

At normal incidence, Fresnel reflections off the glass-sample interface can cause significant measurement artifacts, so the sample plane was tilted by about 45 deg with respect to the optical axis to prevent specular reflections from entering the detection path. A significant portion of the illumination beam still coupled into the detection path and was found to be due to Fresnel reflection from one of the beamsplitter faces. This signal, however, remained constant during the measurement process and was mathematically removed by measuring a background spectrum, $I_{bg}(\lambda)$, with no sample present. A block of Spectralon (Labsphere, Inc., North Sutton, New Hampshire), a spectrally flat thermoplastic resin, was used to collect a reference spectrum, $I_{ref}(\lambda)$. At each pixel location, the remitted scatter spectrum (I_R) was calculated by dividing

the measured spectrum (I_{meas}) by the reference spectrum (I_{ref}) to remove the instrumental spectral response, as shown in the equation:

$$I_R(\lambda) = \frac{I_{meas}(\lambda) - I_{bg}(\lambda)}{I_{ref}(\lambda) - I_{bg}(\lambda)}. \quad (1)$$

The raster-scanning and spectral acquisition sequence is synchronized and controlled by a custom-developed LabVIEW front end. The scan length is limited to 25.4 mm in both directions, providing a full field capability of 254×254 pixels at 100- μm resolution.

2.2 Parameter Fitting

In the absence of significant local absorption, the relationship between the measured irradiance and wavelength would be approximated by a power law-type empirical relation:

$$I_R(\lambda) = A\lambda^{-b}, \quad (2)$$

where A is the scattered amplitude, and b is the scattering power. However, in the presence of significant local absorption, for very small source-detector separations, an empirical relationship can be used to estimate the spectral reflectance:

$$I_R = A\lambda^{-b} \exp[-kc\{d[\text{HbO}_2(\lambda)] + (1-d)\text{Hb}(\lambda)\}], \quad (3)$$

if scattering and absorption coefficients are within the typical range found in tissue.^{29,30} Parameter c is proportional to the concentration of whole blood, k is the path length, and d is the hemoglobin oxygen saturation fraction. The extinction spectra of oxygenated and deoxygenated hemoglobin, HbO_2 and Hb , were obtained from the Oregon Medical Laser Center database.³² This data has a spectral resolution of 2 nm, and a standard piecewise cubic Hermite interpolation polynomial was used to estimate extinction values for in-between wavelengths. Since the absorption from other chromophores is minimal in the waveband of interest, their contributions were assumed to be negligible.

The spatial extent of illumination and detection was less than one mean free path length of interaction (typically 100 μm), so the detected photons are thought to be predominantly singly scattered, and the measured spectrum is independent of local absorption. However, in regions where high local concentration of chromophores is encountered, like regions with blood pooling or high vascularity, this approximation is not valid, and a correction for absorption is required to describe the scatter spectrum accurately. The exponential term in Eq. (3) serves as this correction factor, with the empirical fitting process providing an estimate on the product of the path length and the whole blood concentration, kc and the hemoglobin oxygen saturation fraction d . It should be noted that, in this probe geometry, it is not possible to decouple the path length k and the absolute chromophore concentration c from the measured spectra.

Another parameter of interest is the average scattered irradiance, I_{avg} , integrated over all wavelengths and given mathematically by:

$$I_{avg} = \int_{\lambda_1}^{\lambda_2} I_R(\lambda) d\lambda. \quad (4)$$

Only a subset of the measurement waveband, from 610 nm to 785 nm, was considered for computing I_{avg} , where absorption due to hemoglobin, the dominant chromophore, is minimal. This parameter provides a quick and direct estimate of average scatter without the need for an empirical model and is directly proportional to the irradiance that would be detected by a simple camera without wavelength filtering.

2.3 Animals

All animals were housed in the animal facility with 12-h light–dark cycles, with temperature control, free access to water, and standard laboratory diet. All procedures and experiments were approved by the Animal Care and Use Committee of Dartmouth College.

2.4 Prostate Tumors

Orthotopic prostate tumors were grown in male Copenhagen rats (average 120 to 150 g body weight, Charles River Laboratories, Wilmington, Massachusetts).³³ For all procedures, the rats were anesthetized with an intramuscular injection of a mixture of ketamine and xylazine (90:9 mg/kg). For orthotopic implants, the rat abdominal area and hind flanks were shaved and prepared in an aseptic surgical manner. R3327 MatLyLu Dunning prostate cancer cells³⁴ were cultured in RPMI 1640 with glutamine (Mediatech, Herndon, Virginia) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, Utah) and 100 units/mL penicillin-streptomycin (Mediatech, Herndon, Virginia). The tumor was induced by injection of 1×10^5 cells on the right flank in 0.05-mL sterile phosphate buffered saline (PBS). Tumor growth was assessed by external measurements with calipers, three times per week. Tumors were used for the experiment at 9 to 12 days after inoculation, with a surface diameter of 7 to 9 mm and a thickness of 2 to 4 mm.

2.5 Pancreatic Tumors

For the subcutaneous pancreatic tumors, male C.B.-17 strain 236 SCID mice (6 to 7 weeks, Charles River Laboratories, Wilmington, Massachusetts) were used. Human pancreatic tumor cells, AsPC-1, were grown and maintained in RPMI 1640 with 2-mM L-glutamine with 25-mM HEPES (Lonza, Walkersville, Maryland), and 1.0-mM sodium pyruvate and fetal bovine serum, 10% (Atlanta Biologicals, Lawrenceville, Georgia), with 100 units/mL of penicillin-streptomycin (Mediatech, Herndon, Virginia). Tumors were induced by injecting 1×10^6 AsPC-1 cells (50 μ L volume) subcutaneously in the flank region. Cells were suspended in a solution of 50% culture medium (without FBS) and 50% Matrigel (BD Biosciences, Bedford, Massachusetts), loaded into insulin syringes, and kept on ice until they were injected under the skin. Tumors were measured (calipers) weekly throughout the seven week study. When harvested for this experiment, the tumors measured 6 to 7 mm in diameter and 5 to 6 mm in thickness.

Table 1 Histopathology-based classification of tissue subregions.

Group	Subgroups
Epithelium	High proliferation index (HPI) tumor cells—High nucleus-to-cytoplasm ratio and high cellular density
	Low proliferation index (LPI) tumor cells—Lower nucleus-to-cytoplasm ratio and lower cellular density compared to HPI tumor cell regions
Fibrosis	Regions exhibiting significant fibrosis subclassified based on the maturity of the fibroplastic response: early fibrosis, intermediate fibrosis, and mature fibrosis
Necrosis	Exudative necrosis—Exudate fluids with dead cells
	Focal necrosis—Spots of dead cells spread out in a viable tumor region

2.6 Tumor Imaging

Excised whole tumors were dissected into 4 to 5-mm-thick sections and imaged using the scatter imaging system. The scan resolution was maintained at 100 μ m, and the measurements were referenced to a Spectralon standard as before. The sample was held inside a rubber gasket and sandwiched between two glass slides, with the imaged surface facing down. This arrangement kept the sample in a reasonably airtight environment and also ensured that a flat surface was presented to the scanning beam. The scanning sample stage took approximately 1 h to scan the entire field of interest. A few drops of PBS were placed on the sample before the start of the scan to prevent it from drying during the measurements. Addition of PBS did not alter the signal in a significant manner. It was assumed that the sample optical properties did not change significantly during the measurement process. After the measurement, the sample was immediately placed in 4% neutral buffered formaldehyde solution for fixation and routinely processed for histology evaluation. All histology sections were superficially cut at 4 μ m and stained with hematoxylin and eosin (H&E) for imaging analysis.

2.7 Histopathology Classification of Tumor Subregions

A veterinary pathologist (P. J. H.) examined the H&E sections from each sample and identified several regions-of-interest corresponding to the tissue types observed. The tissue types observed across all samples were classified under three major groups with constituent subgroups, as listed in Table 1. The epithelial group was identified as containing two distinct types of epithelial cells, one that exhibited higher nucleus to cytoplasm ratio compared to the other. As higher nucleus-to-cytoplasm ratio is indicative of cell proliferation, the subgroup containing this type of cells was identified as the *high proliferation index* (HPI) tumor cells. The subgroup containing cells with comparatively lower nucleus-to-cytoplasm ratio

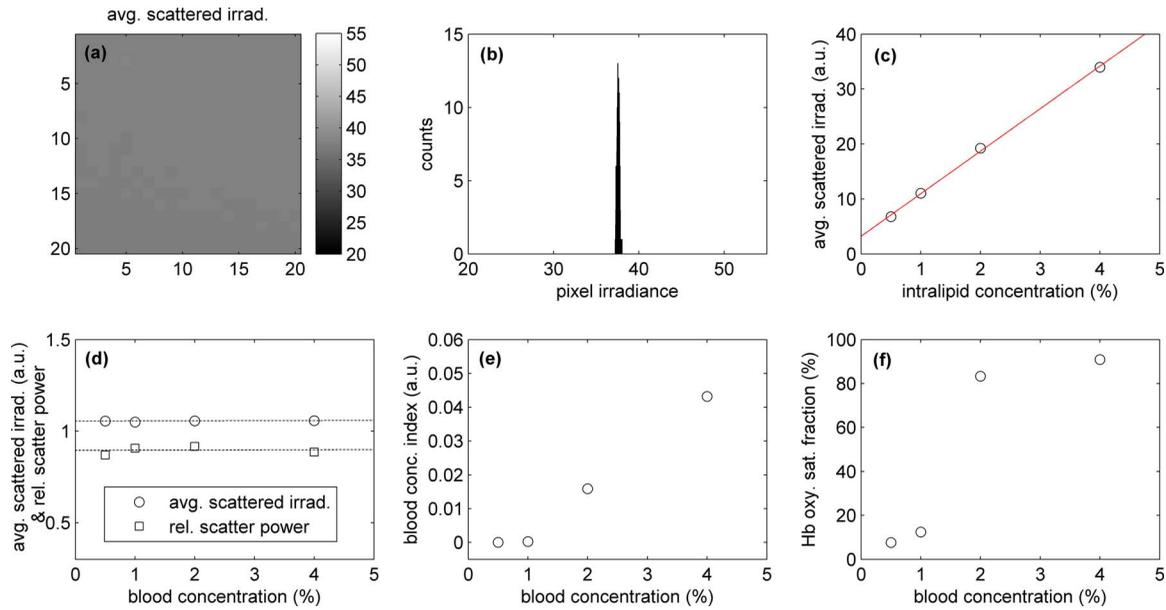


Fig. 2 (a) and (b) Average scattered irradiance image of a 1 cm \times 1 cm homogeneous 5% IntraLipid phantom and its associated histogram. (c) Plot of average scattered irradiance as a function of IntraLipid concentration. (d) Plots of average scattered irradiance (values scaled by a factor of 0.1) and scatter power as a function of blood concentration in a 1% IntraLipid phantom. The estimated scatter parameters are shown to be relatively independent of the changes in blood concentration. (e) Plot of estimated blood concentration index, kc , as a function of blood concentration. (f) Plot of estimated hemoglobin oxygen saturation fraction, d , in phantoms with different concentration of blood.

was identified as the *low proliferation index* (LPI) tumor cells. Regions exhibiting significant fibrosis were classified into early, intermediate, and mature fibrosis subgroups based on the maturity of the tumor's fibroplastic response. *Exudative necrosis*, necrosis marked by the presence of exudative fluids, and *focal necrosis*, spots of dead cells found within a viable tumor region, were the two types of necrotic regions observed in the measured samples.

3 Results

3.1 Phantom Measurements

Since the scatter imager employed a confocal detection scheme, fluctuations in the sample plane's z -position could cause fluctuations in the measured signal. A homogeneous 5% IntraLipid phantom obtained by diluting a stock 20% IntraLipid solution (20% phospholipid-stabilized soybean oil in water) was used to test the spatial uniformity of the scanner over a 1 cm \times 1 cm scan area. Figures 2(a) and 2(b) show the average scattered irradiance image and its histogram. A batch of phantoms of varying volume concentrations of the stock IntraLipid solution was used to test the linear response of the system. The system response, as shown in Fig. 2(c), suggests that in the absence of absorption, the average irradiance of the remitted light is approximately proportional to the reduced scattering coefficient.

To test the probe's insensitivity to local absorption changes, another batch of IntraLipid phantoms was prepared with varying volume concentrations of whole animal blood. Heparinized whole animal blood harvested from a sheep was used, and the IntraLipid concentration was fixed at 1% ($\mu_a = 2.3 \times 10^{-4} \text{ mm}^{-1}$ and $\mu'_s = 1.1 \text{ mm}^{-1}$ at 633 nm, scaled from reported values for IntraLipid 10%).³⁵ The estimated

scatter parameters were expected to remain constant across this batch of phantoms despite the change in absorption. As expected, the average scattered irradiance and scattering power remained constant over increasing whole blood concentration, as shown in Fig. 2(d). Estimated blood concentration index (kc) as shown in Fig. 2(e) was negligible for low concentrations of blood. This behavior at low blood concentrations is expected to be due to the inherent insensitivity of the probe to local absorption. However, for blood concentration values beyond 1%, an approximately linear increase was observed. The estimated hemoglobin oxygen saturation fraction is shown in Fig. 2(f). It should be noted that the estimates of d are not reliable in phantoms with low concentration of blood due to the instrument's insensitivity to absorption under these conditions.

3.2 Tumor Measurements

Six pancreas and four prostate tumor samples were imaged using the scatter imaging system. The average scattered irradiance image and the scattering power images were extracted from the raw spectral images and are shown in Figs. 3(a)–3(d). Figure 4 shows representative spectra acquired from pancreas and prostate tumor samples along with their corresponding empirical fits. Under the guidance of a veterinary pathologist, several regions-of-interest corresponding to tissue types classified in Table 1 were identified on each of the measured tumor samples. Figure 5 shows an example where five regions-of-interest identified in a pancreatic tumor sample are shown overlaid on the scatter power image along with a close-up view (100 \times magnification) of the corresponding H&E sections. Region 1 shows HPI tumor cells with more cellular density compared to LPI tumor cells found in Region 2. Regions 3 and 4 show early and intermediate stages of

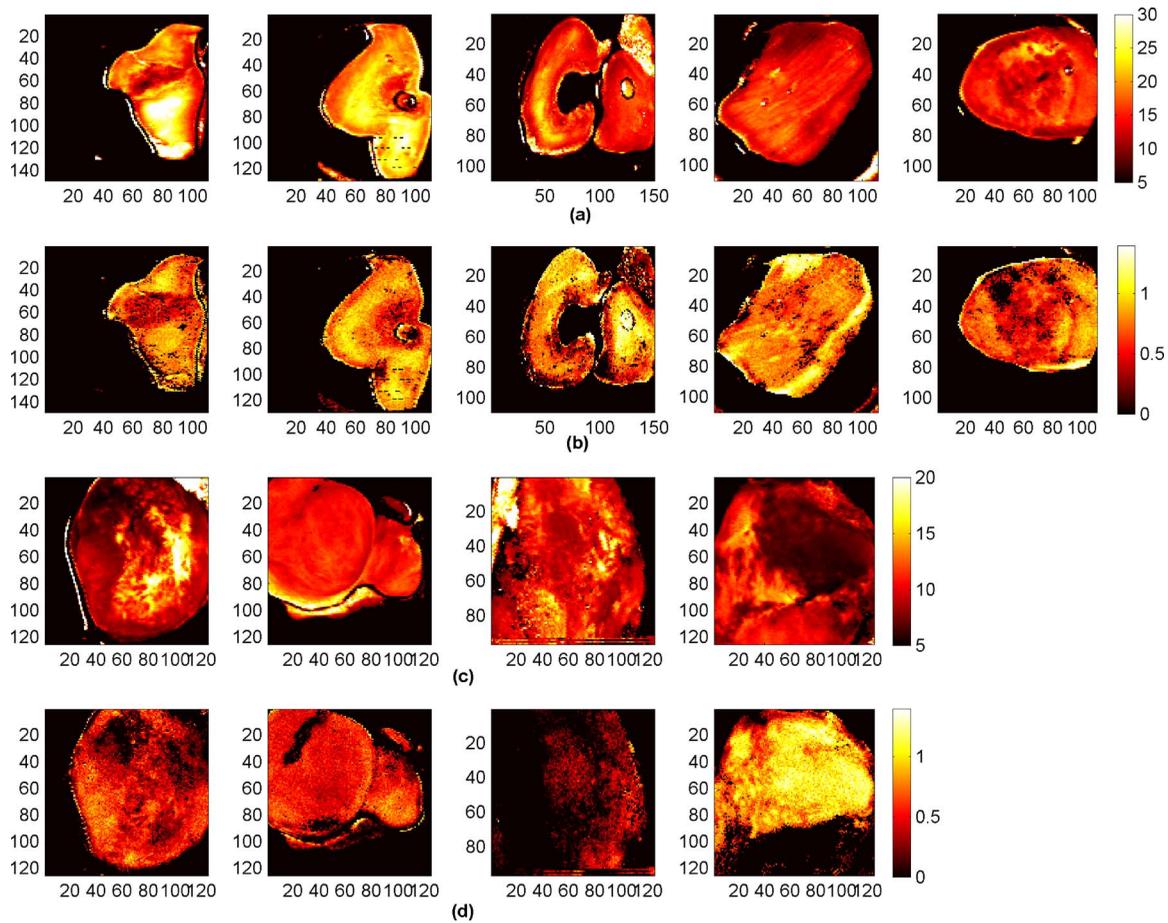


Fig. 3 (a) and (b) Average scattered irradiance and scatter power images of the measured pancreas tumor (AsPC-1) samples. The third image from the left contains two samples that were imaged together. (c) and (d) Average scattered irradiance and scatter power images of the measured prostate tumor (Mat-LyLu) samples.

fibrosis marked by the increased stromal content and less cellularity. Region 5 shows necrosis with the presence of exudative fluids and fragments of dead cells.

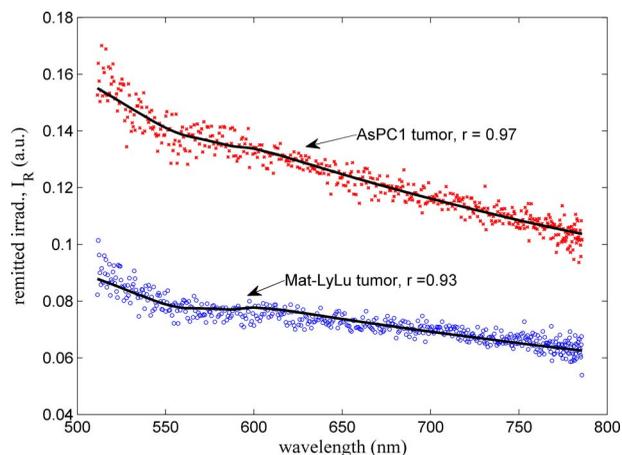


Fig. 4 Representative reflectance spectra measured in pancreas and prostate tumor samples along with their corresponding empirical fits to Eq. (3) (solid line).

The average scattered irradiance and scatter power data for different tissue types were obtained by sampling corresponding histology-guided regions-of-interest in the scatter images. Figures 6(a) and 6(b) show the grouped scatter plots of scattering power versus average scattered irradiance for pancreas and prostate tumor samples. Figures 7(a) and 7(b) show the box and whisker plots of average scattered irradiance and scatter power distributions for different tissue types found in the pancreas tumor samples. Figures 8(a) and 8(b) show the same for the prostate tumor samples. The boxes in these plots contain the “middle 50%” of the data distribution they represent, bounded by the first and the third quartiles. The line within the boxes represents the median (second quartile). The whiskers extend to 1.5 times the interquartile range from either end of the boxes. The notch around the median lines represents the 95% confidence interval within which the “true median” of the represented distribution exists. If the notches in two plotted distributions do not overlap, then one can conclude with 95% confidence that their true medians differ. The + symbols outside the whiskers indicate the outliers.

4 Discussion

The microsampling approach to spectroscopy applied here utilizes the local confinement of the signal to reduce the need

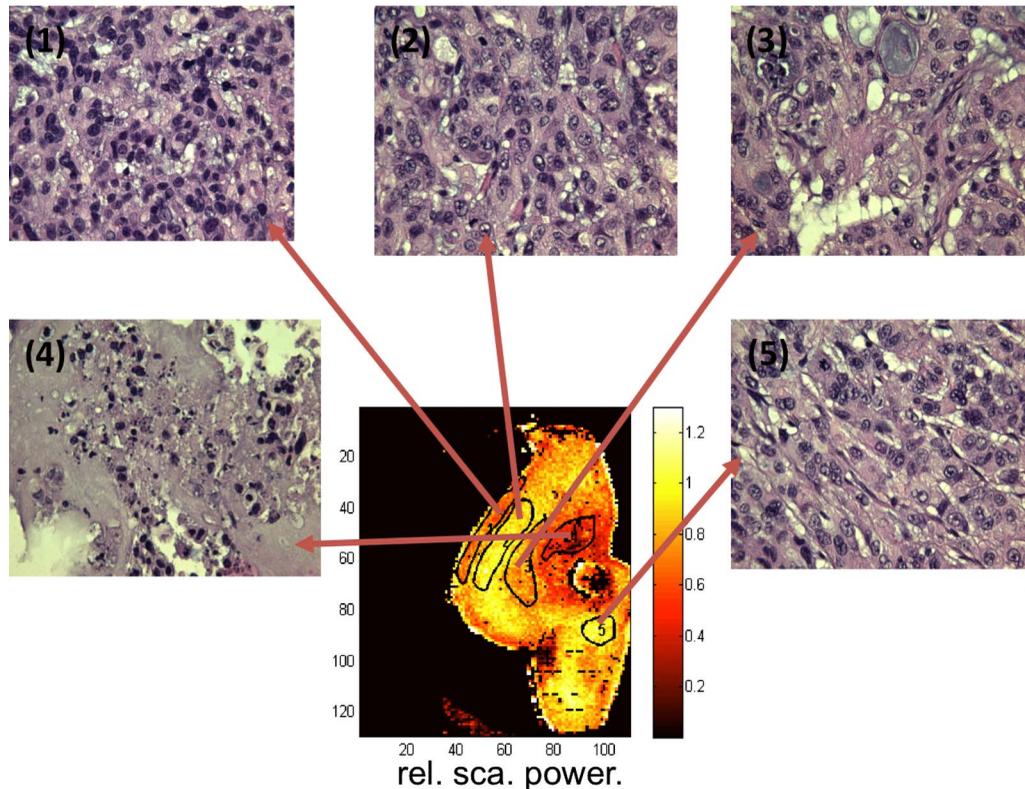


Fig. 5 Scatter power image of a pancreas tumor sample showing five pathology-based regions-of-interest overlaid on it. Variations in tissue ultrastructure are evident on the shown high magnification (100 \times) pathology images: (1) High proliferation index (HPI) tumor cells, (2) low proliferation index (LPI) tumor cells, (3) significant early fibrosis with some lipids, (4) necrosis (exudative), and (5) mature fibrosis.

for complex light transport modeling, which is often needed in deeper tissue signals. The confocal optical system confines the signal to approximately 100 μm laterally and to approximately a few hundred microns in the depth coordinate. The fitting used in Eq. (3), while empirical, has been found to be robust and a useful way to quantify scatter power from the remitted signal. The scatter power is independent of most coupling errors and uniquely associated with features related to the effective scatterer sizes.^{36–38} A key feature in the design of this instrument has been to have a standard reference and acquire the spectrum in a referenced manner, so that measurements between samples could be compared directly for average value differences and for examination of the variation within a “homogeneous” tissue type.

Despite this approach, it is believed that the remitted irradiance is perhaps a less reliable parameter than the scatter power, because this latter parameter is independent of coupling errors in the imaging system. Although all the spectral images acquired by the scatter imaging system were referenced to Spectralon to minimize referencing artifacts, the average scattered irradiance and scatter amplitude images were not entirely free from such artifacts due to small sample positioning and other instrumental issues. The increased “spread” in the average scattered irradiance compared to the scattering power as observed in Figs. 6(a) and 6(b) is consistent with this behavior. As a result of this, high variance was observed in the average scattered irradiance box plots shown in Figs. 7(a) and 8(a). In addition to referencing issues, the scatter amplitude images also exhibited some coupling arti-

facts and hence were considered less reliable. However, the scatter power images were found to provide consistent trends across different tumor samples. This was due to the fact that scatter power relates to the slope of the wavelength-dependent scatter function and hence is relatively free from referencing artifacts.³⁹ Still, interpreting the variation in scatter observed within a single tissue sample clearly indicates that the integrated scatter irradiance can vary up to 50% in most tumor samples, which is a level of heterogeneity that would be significant in optical therapies such as photothermal or photodynamic therapy. These imaging results can be used to better appreciate that there is a clear need for *in situ* light dosimetry in photodynamic therapy, since this high heterogeneity level will lead to significant variations in the tissue being treated. Further analysis of the effect that this variation has upon the transmitted light irradiance is clearly warranted.

However, for diagnostic purposes, one additional conclusion is that the scatter power may be a more reliable metric to compare between different samples, as it eliminates errors related to the coupling of the tissue to the microscope imager. The scattering power distributions of various tissue types plotted in Figs. 7(b) and 8(b) were compared. The median scatter power of proliferative epithelium was found to be about 26% less than the same for mature epithelium in both pancreas and prostate tumor samples. It is well known that a significant portion of light scattered from tissue at higher scattering angles (>40 deg) comes from nuclear features and intracellular organelles in cytoplasm such as mitochondria,^{40–44} although recent work also shows that the stroma between the

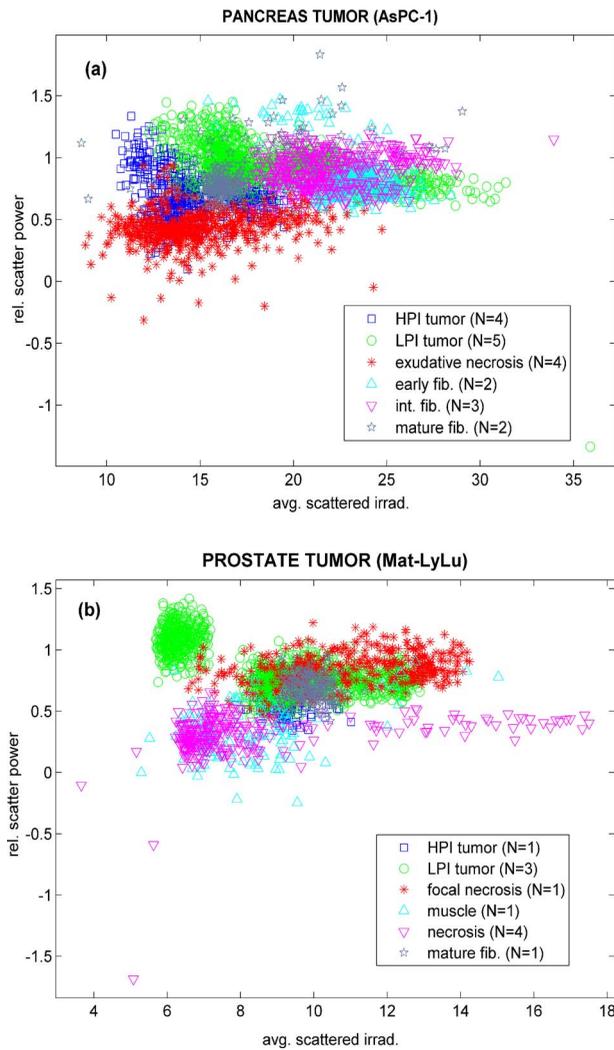


Fig. 6 (a) and (b) Grouped scatter plots of scattering power versus average scattered irradiance for all significant tissue types identified in pancreas and prostate tumor samples.

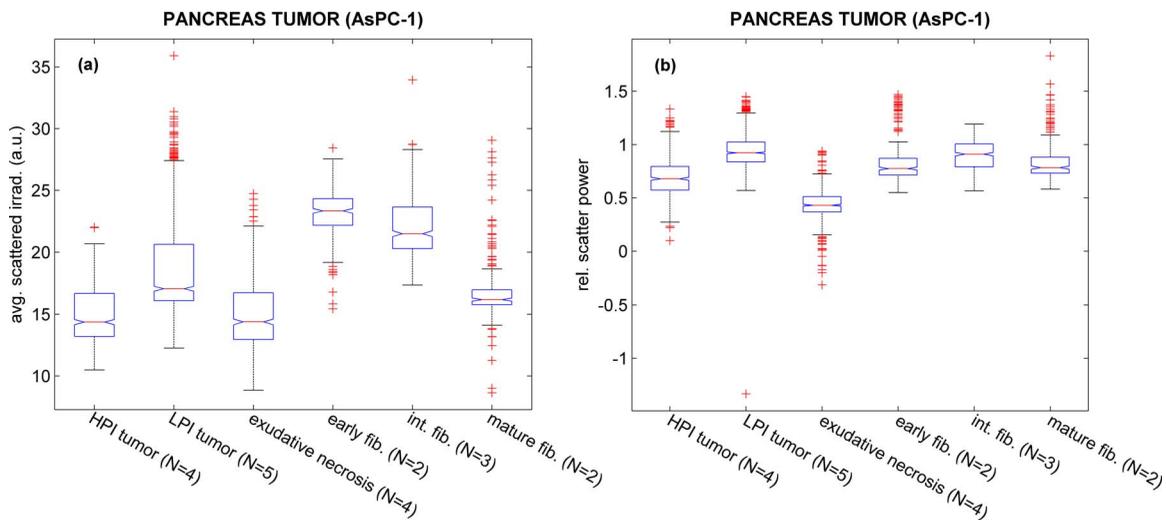


Fig. 7 Box and whisker plots of (a) average scattered irradiance and (b) scatter power distributions, for all significant tissue types identified in pancreas tumor samples.

cells can influence the scattering signature significantly.⁴⁵ Histopathology observations on the measured tissue types in this study revealed that LPI tumor cells exhibited higher cytoplasmic content and lower cell density, as compared to the HPI tumor cells, which contained bigger nuclei and were more tightly packed. The higher organelle density per unit volume seen in mature tumor cells, due to increased cytoplasmic content, would clearly lead to higher scatter power as the number of small scattering features increases.

Exudative necrosis exhibited the lowest scattering power response among all tissue types considered, with its median scatter power about 53% less than that of LPI tumor cells in pancreas tumor samples and 59% less than that of LPI tumor cells in prostate tumor samples. This decrease has a physical explanation, since the density of cells is significantly decreased and inflammatory infiltrate (edema, extracellular water, or protein solute) occupies much of the space where tumor cells used to be. In comparison to exudative necrosis, regions of focal necrosis primarily contain mature tumor cells with spots of dead cells interspersed, and the scattering from these latter regions was similar to the mature tumor cell regions. The median scatter power of these two regions differed only by about 6%. Thus, the presence of the focal necrotic regions did not seem to have a significant reduction in the overall scatter. However based upon the reduction seen with exudative necrosis, it might be hypothesized that the scatter signal reduces in proportion to the fraction of cellular content within the region.

The prostate tumor samples contained only one region exhibiting significant mature fibrosis. The median scatter power in that region was about 13% lower than that of similar regions found in the pancreas tumor samples. Within the pancreas tumor samples, no significant difference in scatter power was observed between mature and early fibrosis regions. The intermediate fibrosis region was found to scatter more, with about 16% increase in the median scatter power. It is more challenging to interpret what these numbers mean, if anything, about how the morphology relates to the light scattering

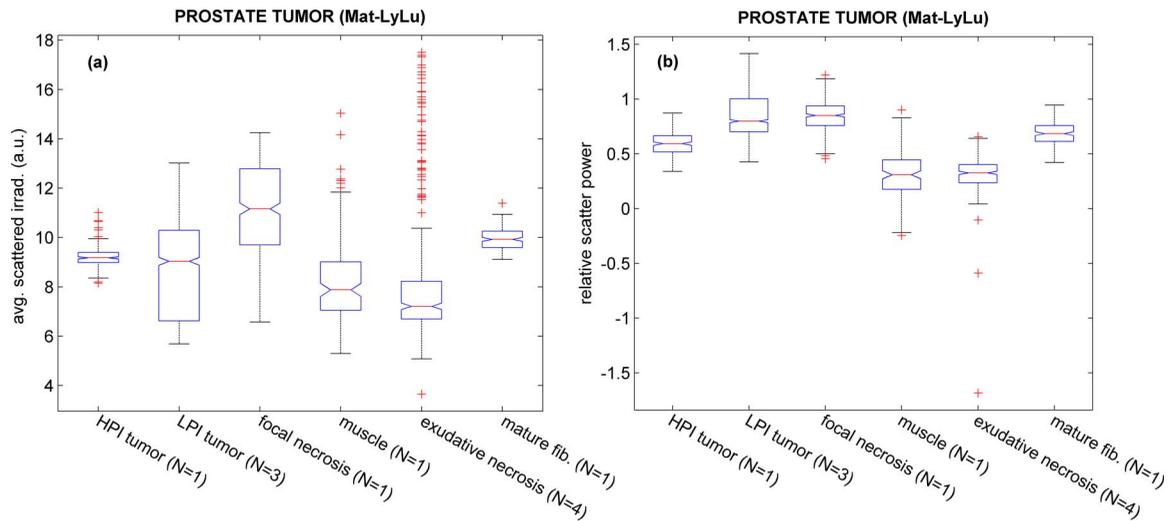


Fig. 8 Box and whisker plots of (a) average scattered irradiance and (b) scatter power distributions, for all significant tissue types identified in prostate tumor samples. No regions corresponding to early and intermediate fibrosis were identified in the prostate tumor samples.

response. Since the changes are so subtle, it is likely difficult to quantify this further.

Finally, in all the measurements discussed earlier, the acceptance angle defined by the detection optical train limits the angular region sampled in the backscatter geometry. So it should be noted that the scatter signal measured here is actually a complex mixture of both the scattering spectrum and the scatter phase function spectrum. Changes in the phase distribution of scattering could have significant influence on the discussed measurements. A comprehensive study on the effects of phase in these measurements would require discrete sampling of a significant portion of the backscatter hemisphere and is beyond the scope of this study, although this is being studied in setups that are more conducive to this problem.^{44,46–48} Unfortunately, the problem of measuring angularly resolved data from bulk tissue is complex, and so sampling of scatter from a narrow angle of collection is likely the only practical way to sample the scatter spectrum in thick tissues.

5 Conclusions

This study reports on the development of and data collection from a raster-scanning confocal reflectance imaging system to directly quantify tissue scatter changes *in situ* with an attempt to interpret these changes relative to the tissue morphology. Phantom studies were conducted to validate the instrument performance and to demonstrate its insensitivity to local absorption changes. Last, pancreas and prostate tumor samples were characterized using this instrument. The results, in combination with standard histopathology, demonstrate the instrument's capability to quantify scatter power and that the scatter power appears correlated with the epithelial morphology. It is not clear how the signal-to-noise ratio of imaging scatter power will compare to that of imaging average scattered irradiance. The latter is analogous to what would be seen by the human eye, but scatter power would be analogous to the color spectrum of the scattered irradiance. The instrument is currently limited to *ex vivo* measurements due to inherent limi-

tations of the sample scanning architecture and low scan speeds. The instrument's utility can be extended by moving to a beam scanning architecture that would be rapid and hence more suitable for measurements in clinical settings.

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