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Monochromatic subdiffusive spatial frequency domain imaging provides *in-situ* sensitivity to intratumoral morphological heterogeneity in a murine model

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Abstract

For the first time, spatially resolved quantitative metrics of light scattering recovered with sub-diffusive spatial frequency domain imaging (sd-SFDI) are shown to be sensitive to changes in intratumoral morphology and viability by direct comparison to histopathological analysis. Two freshly excised subcutaneous murine tumor cross-sections were measured with sd-SFDI, and recovered optical scatter parameter maps were co-registered to whole mount histology. Unique clustering of the optical scatter parameters μ'_s vs. γ (i.e. diffuse scattering vs. relative backscattering) evaluated at a single wavelength showed complete separation between regions of viable tumor, aggressive tumor with stromal growth, varying levels of necrotic tumor, and also peritumor muscle. The results suggest that with further technical development, sd-SFDI may represent a non-destructive screening tool for analysis of excised tissue or a non-invasive approach to investigate suspicious lesions without the need for exogenous labels or spectrally resolved imaging.

Keywords

scatter imaging; tissue characterization; SFDI

1. Introduction

Diffuse optical measurements of tissue can provide sensitivity to physiology, metabolism, and structure over wide fields of view. Such information is obtained by spectroscopic and/or spatial analysis of radiant photon propagation and attenuation. Model-based interpretation of diffuse remission uses the simplifying assumption that multiply scattered light can be described using isotropic reduced scattering. However, diffuse measurements offer limited sensitivity to microscopic heterogeneity in tissue composition due to volumetric averaging over tortuous photon path lengths. Conversely, sub-diffusive measurements that collect remission close to the source of entry provides sensitivity to anisotropic aspects of scatter direction. Directional preferences in scatter are dictated by the microscopic distribution of mass density in the sampled tissue, and in turn, are sensitive to the density, organization, and

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fractal dimension of structural features within biological tissue [1–3]. Techniques utilizing confocal [4], fiber based [5], or interferometric [6] methods are capable of localizing reflectance remission to the superficial layers of tissue and preserving sensitivity to anisotropic remission. Such measurements are capable of sensing variations in cellular internal machinery (e.g. nuclear morphology) and/or extracellular matrix (e.g. stromal density) that are considered hallmarks of the onset of cancer [7]. However, adapting these approaches to image variations in tissue morphology for clinical applications is a challenge, as raster-scanning these microscopic point-sampling approaches over large fields of view is cumbersome and time consuming. Recently, sub-diffusive spatial frequency domain imaging (sd-SFDI) was shown to provide sensitivity to superficial, weakly scattered photons over a wide-field geometry, and provide images of tissue microarchitecture that may be relevant for surgical guidance [8].

Previous investigations of sd-SFDI have used model-based analysis of multiple spatial frequencies sampled over multiple wavelengths to recover quantitative scattering parameter maps [8–10]. This parameter set includes the reduced scattering coefficient, μ'_s , and a phase

function parameter, $\gamma = \frac{1 - g_2}{1 - g_1}$, which characterizes the backscatter probability of the scattering phase function, and is expressed as a weighted ratio of the first two Legendre moments of the distribution of scattering angles, g_1 and g_2 [11]. The reduced scattering coefficient, μ'_s , quantifies the frequency of isotropic scattering, which is related to the density of scattering features, weighted by their effective scattering cross sections and the relative directionality of scattering. The γ parameter represents an implicit metric of the size-scale distribution of scattering features that are relatively large or small compared to the wavelength of light. This parameter can indicate contributions from larger Mie-type scatterers, which tend to scatter predominantly in the forward-direction, or smaller Rayleigh-type scatterers, which scatter more isotropically [12]. Furthermore, it has been numerically and experimentally shown that γ is proportional to the fractal dimension of a distribution of spherical scatterers [8, 12]. In biological tissues, these sub-diffusive scattering parameters have been identified as a basis for differentiating morphological changes between malignant and normal human brain tissues [13] and also human breast tissues [8]. Previously, our group employed sd-SFDI to show significant changes in scattering parameters between aggregate cancerous and normal tissues [8]. The present proof-of-principle study considers variations in sub-diffusive parameters observed within malignant tissues. Specifically, the study considers how spatial maps of μ'_s and γ extracted at a single wavelength of light show spatial clustering that is associated with variations in aspects of tumor morphology that are extracted from quantitative histological analysis. This pilot study uses a few samples (n=2) to demonstrate the sensitivity of these sub-diffusive parameter maps to changes in tissue ultrastructure within a given tumor specimen.

2. Experimental Methods

While a complete description and validation of sd-SFDI can be found in previous work [8], a concise summary is described below. A modified commercial spatial frequency domain imaging system from Modulated Imaging, Inc. (Irvine, CA) [14] with a maximum field of

view of roughly $10.5 \text{ cm} \times 14 \text{ cm}$, sampling resolution of $200 \text{ }\mu\text{m}$, and projection resolution of $150 \text{ }\mu\text{m}$ was used for imaging. The basic setup of the camera and projector is shown in Figure 1, where imaging is done in an inverted geometry through a glass slide in order to mitigate surface curvature artifacts of the sample, arising when the sample is non-normal to the optical axis. Although these artifacts pose a challenge for *in-vivo* translation, recent work aims to mitigate their affects [15]. The digital micromirror based projection device is coupled to multiple LEDs as light sources. To emphasize translation, this study reports a monochromatic analysis at the 658 nm source, which corresponds to a red channel in a commercial surgical microscope, which are commonly employed for surgical resection of tumors. Cross-polarizers were removed to provide sensitivity to weakly scattered photons.

The structured illumination patterns were sinusoids of the form $I(x, y, f_x) = 0.5[\sin(2\pi f_x x + \Phi_i) + 1]$ with $\Phi_i = [0, \frac{2\pi}{3}, \frac{4\pi}{3}]$ where $I(x, y, f_x)$ represent the image stack of sinusoids with spatial frequency, f_x sampled over 18 linearly spaced values between 0 and 0.85 mm^{-1} , for three phase offsets, $i=[1, 2, 3]$, used for demodulation. While spatial frequencies where $\mu'_s/f_x \leq 3$ have strong sub-diffusive sensitivity, it may be possible to use fewer non-linearly spaced spatial frequencies; however, a full analysis of spatial frequency sensitivity to each optical property is beyond the scope of this letter and is also the subject of a recent study by Bodenschatz *et al* [16]. The demodulated reflectance, $I_d(x, y, f_x)$ was calculated with the

following formula: $I_d(x, y, f_x) = \frac{\sqrt{2}}{3} \sqrt{(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2}$.

The Modulated Imaging system produced a 16-bit image per acquisition, and MATLAB (Mathworks, Inc) was used to read in and demodulate the raw images. Demodulated images measured in tissue samples were referenced through a normalization by a liquid phantom containing 1% Intralipid (IL). Because the illumination source was not collimated and the cross polarizers were removed, measurements of a non-turbid phantom containing water were used to estimate specular reflection within the imaged field of view. These demodulated images of water were subtracted from both the sample and IL as a dark reference. These normalized and referenced raw sample images were then calibrated by a model predicted reflectance value for the 1% IL normalization phantom. The optical properties of IL were calculated according to Michels *et al*. [17] with an estimated batch to batch variation in reduced scattering of less than 2% as shown by Ninni *et al*. [18]. For spatial frequencies below 0.2 mm^{-1} a diffusion theory model is used [14], and for spatial frequencies above 0.5 mm^{-1} a sub-diffusive model is used [9].

$$R_d(x, y, f_x) = R_{\text{Model}}(f_x) \frac{I_d(x, y, f_x) - I_{d_{\text{water}}}(x, y, f_x)}{I_{d_{\text{IL}}}(x, y, f_x) - I_{d_{\text{water}}}(x, y, f_x)}$$

$$R_{\text{Model}}(f_x) = \begin{cases} R_{\text{Diff}} & \text{if } f_x \leq 0.2 \text{ mm}^{-1} \\ R_{\text{SubDiff}} & \text{if } f_x \geq 0.5 \text{ mm}^{-1} \end{cases}$$

Calibrated reflectance intensity images, $R_d(x, y, f_x)$ were inverted to maps of μ_a , μ'_s , and γ using a non-linear least squares minimization. The difference between the log of model predicted reflectance and log of experimental reflectance was minimized on a pixel-by-pixel basis, with μ_a , μ'_s , and γ at the imaged wavelength being the fit variables. The MATLAB *lsqnonlin* routine was utilized with the *levenberg-marquardt* algorithm and the fit variables bound to a positive real set. This model and inversion algorithm was extensively validated with both simulated and experimental data [8]. Optical properties of simulated Monte Carlo data with $\mu'_s = [0.3 - 10] \text{ mm}^{-1}$ and $\gamma = [1.3 - 1.9]$ for a Modified Henyey-Greenstein phase function and also with $\gamma = [1.2 - 2.4]$ for a Mie Theory phase function were accurately recovered with the inversion algorithm [8]. For experimental validation, fractal distributions of polystyrene spheres with $\mu_a = [0.02 - 0.18] \text{ mm}^{-1}$, $\mu'_s = [1.2 - 2.4] \text{ mm}^{-1}$, and $\gamma = [1.28 - 1.68]$ at 658 nm were imaged and accurately inverted. Three experimental validations were performed: constant μ'_s with γ variation, both μ'_s and γ variation, and both μ_a and γ variation. Recovered optical property maps were median filtered with a 10×10 pixel ($2 \times 2 \text{ mm}$) kernel to remove noise. Analysis of spatially heterogeneous step-change phantoms characterized the optical resolution, defined as the length over which 75% of the maximum contrast of the step change has been reached, to be $2000 \text{ } \mu\text{m}$ (10 pixels) for μ'_s and $800 \text{ } \mu\text{m}$ (4 pixels) for γ . [8].

The sd-SFDI setup was used to image cross-sections of a murine subcutaneous breast tumor model. All animal procedures were conducted under a protocol approved by the Dartmouth College Institutional Animal Care and Use Committee (IACUC) in accordance with all Personal Health Service (PHS), federal, Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and institutional guidelines. The tumor line used for this study was a mouse mammary adenocarcinoma/MTG-B. Tumor cells were implanted intradermally ($1 \times 10^6 \text{ cells}$) in the flank region and were allowed to grow to a volume of approximately 650 mm^3 . When tumors reached this volume, the mice were humanely sacrificed, and the tumors were excised and bisected into two equal hemispheres. The tumor hemispheres were then placed cut surface down on a glass slide and imaged as shown in Fig 1 (a). This *ex-vivo* imaging procedure was completed within 5 minutes of excision. The tissue sections were then fixed in 4% neutral buffered formaldehyde, embedded in paraffin, and cut to a $4 \text{ } \mu\text{m}$ thick histologic section. The resulting slides were stained with hematoxylin and eosin (H&E). The sections represented a well-approximated anatomical match to the sd-SFDI optical scatter parameter maps, limited only by distortions brought on by histological processing of the tissue. A specialty trained veterinary pathologist (PJH) provided detailed morphological descriptions of various regions within in tissue section. Areas of high and low grade tumor were holistically determined based on morphological features such as cell differentiation, nuclear to cytoplasmic ratio, and production of extra-cellular collagen. These sections were co-registered to the scatter parameter maps for cluster based analysis.

3. Results

Figure 2 presents optical imaging data for two tumor cross-sections and correlation between the histopathological and scattering parameters. Color photographs in Fig. 2(a1 & b1) and full-field H&E sections in Fig. 2(a4 & b4) present clearly discernible differences in morphology both between and within the two tumors. Regional inspection of the H&E reveals a link between color-coded microscopic features in Fig. 2(a5–a7 & b5–b8) and the recovered optical property maps in Fig. 2(a2–a3 & b2–b3). Clusters of pixels from each region showing separation on the μ'_s vs. γ plot in Fig. 2(a9 & b9).

Tumor A shows a highly necrotic core region surrounded by solid low grade tumor in the white light and full field H&E images, Fig. 2(a1 & a4) respectively. Low grade tumor cells, located in regions near the tumor boundary in Fig. 2(a6) (cyan), are intermixed with diffusely distributed necrotic tissue in Fig. 2(a5) (magenta). However, in Fig. 2(a7) (red) there is primarily necrotic tissue that has retained seminormal tumor morphology. The necrotic tumor region in Fig. 2(a8) (blue), which appears as a void on the H&E, exhibited lower γ values, associated with primarily Rayleigh scattering. A decrease in scatter density (μ'_s) is shown between the still-solid region in Fig. 2(a7) (red) and the liquid/necrotic region in Fig. 2(a8) (blue).

Tumor B shows high-grade morphology in the center region with neighboring areas of lower grade tumor, necrosis, and muscle. This appears as white in the center with an increase in transparency near the less dense tumor and muscle, while the area of necrosis shows a slight reddening. Fig. 2(b5) (purple) shows peritumor muscle characterized by large aligned myofibrils; these properties relate to a low μ'_s and high γ that are representative of large Mie-type scatterers. Fig. 2(b6) (yellow) shows high-grade viable malignant cells near the tumor border with aggressive stromal growth. The region identified in Fig. 2(b7) (cyan) is also on the tumor border, but appears to be of a lower grade with less voluminous proliferation as compared with Fig. 2(b6) (yellow). Another largely necrotic region is identified in Fig. 2(b8) (red), again with similar semi-normal tumor morphology. Whereas the viable tumor has a normal morphology.

In Fig. 3 (a), thumbnails of viable tumor sections from both tumors are shown with the extra-cellular matrix (ECM) highlighted green through a color based segmentation done in MATLAB. In short, the images of viable tumor are converted from RGB space to HSV space, thresholds for hue, color saturation, and brightness are applied to isolate extracellular collagen based on its unique affinity for the eosin stain, and finally the fraction of segmented pixels is calculated. In Fig. 3(b & c), γ and μ'_s are plotted versus the fraction of ECM in the respective histologic sections. While in Fig. 3(d), thumbnails of various sections from both tumors are shown with the areas of low-grade viable tumor shaded yellow and areas of necrosis shaded green, and Fig. 3(e & f), γ and μ'_s are respectively plotted versus the fraction of necrosis in the histologic sections.

The effect of stromal proliferation on optical properties for low grade tumor (cyan) and high grade tumor (orange) is shown in Fig. 3(b & c). With an increasing fraction of ECM, optical

parameters showed an increase μ_s' ($r=0.947$) that are representative of densely packed scattering structures, and a decrease in γ ($r=-0.884$) as the stromal collagen acts like a Rayleigh-type scatterer with refractive index fluctuations much smaller than the wavelength of light. These correlations suggest that stromal proliferation, which is related to tumor grade, has a strong effect on optical properties. However, it is important to note many other ultrastructural changes on length scales much smaller than the diffraction limit also have profound impact on the optical properties. The transition in optical properties from the low-grade tumor sections (cyan) to the mixed necrotic section (magenta) and totally necrotic sections (red) is shown in Fig. 2(e & f), with an increase in scatter frequency (i.e. increase in μ_s' as function of % necrosis, $r=0.993$) and decrease in Mie-forward scattering (i.e. decrease in γ as function of % necrosis, $r=-0.906$). A possible explanation for this trend in optical properties is as the cells lyse during necrosis, the individual Mie regime cells, nuclei, and mitochondria become a chaotic suspension of Rayleigh regime proteins.

4. Discussion and Conclusions

The data in Fig. 2 and 3 show that quantitation of light scattering provided by sd-SFDI reveals information about tissue microstructure that is not readily discernible from qualitative inspection of white light imaging. Reflectance imaging over multiple spatial frequencies allows independent estimation of metrics of scatter frequency (i.e. μ_s') and scatter size scale distribution (i.e. γ). The present study shows, for the first time, the use of sd-SFDI to quantify spatial variations in microarchitecture of tumors highlighting the sensitivity of the measurement to changes in structure associated with tumor grade and viability. This study also demonstrates the use of sd-SFDI at a single wavelength, reducing the requirements for spectrally resolved acquisition and potentially enhancing the ability to translate the approach to existing surgical imaging equipment.

Previously reported validation studies have demonstrated accurate quantification of each sub-diffusive parameter independently, with systematic variation in both the density and the size scale distribution of scatterers and also with variations in background absorption [8]. To investigate the independence of these two parameters in biological media, the Pearson Product correlation coefficient was calculated for μ_s' and γ on a pixel-by-pixel basis for the full image of Tumor A ($r=-0.56$) and Tumor B ($r=-0.63$) (data not shown). These results suggest mild cross-talk between the recovered scattering parameters, which can be explained by biophysical correlations in sub-regions of the tumor such as those shown in Fig. 2 & 3, and while such cross-correlations were not observed in phantom data [8], these results do motivate further development the optical property inversion algorithm.

Coupled interpretation of these sub-diffusive scattering parameters, which are recovered at each pixel in the imaged field of view, provides a multi-parametric basis to characterize tissue types. The results presented in this study represent, to the knowledge of the authors, the first quantitative sub-diffusive analysis of internal tumor architecture, and demonstrate the ability of light scattering to detect specific changes in tumor morphology. Results highlight that the biological variability in malignant tissue is substantial, and are consistent with previous claims that the use of scatter imaging for diagnostic evaluation of tumor

margins may require the spatial interpretation of optical maps, as done in Laughney *et al.* [4]. While non-uniqueness of optical properties, *i.e.* multiple tissue type having similar optical properties, presents a limitation of this method, contextual information about the sample can guide the interpretation of the signal as is commonly done in flow cytometry. It also is important to note that the data presented in this study are from a limited representative set of individual tumors, limiting the ability to make meaningful generalizations. Furthermore, orthotopic murine models with diffusely mixed tissues and more aggressive perfusion may provide a more realistic tumor model for clinical applications than the subcutaneous model utilized in this study. Future work is needed with increased statistical power to fully assess the sensitivity of this method, but the comparison of micro-structural morphology with sub-diffusive optical parameters in this study provides insight as a proof-of-principle demonstration into the use of sd-SFDI for multi-length scale imaging of biological tissue. Without the need for exogenous dyes, spectroscopic analysis, or raster scanning, monochromatic sd-SFDI is a promising technique for rapid *in-situ* tissue characterization.

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Figure 1.

Schematic of major components of sd-SFDI device. A red (658 nm) light emitting diode (LED) illumination source reflects off a digital micromirror device (DMD) to spatially modulate the light intensity. The sinusoidal illumination pattern is focused onto the sample, which is lying flat on a glass slide. A charged coupled device (CCD) captures the perturbed intensity patterns, which are used to calculate the optical property maps.

Figure 2.

In (a1) and (b1), photographs of the bisected cut surfaces of two mouse mammary tumors are shown. The γ maps in (a2) and (b2) represent the scatter size scale distribution, while the μ_s' maps in (a3) and (b3) represent isotropic scatter density in the tumor sections. In (a4) and (b4), low magnification composite histological sections of the tumors are shown. The higher magnification images shown in (a5–a7) and (b5–b8) illustrate morphological variation observed within the histological sections. The morphological features include viable tumor, necrotic tumor, cavitated/liquefied tumor, and peritumor muscle. The scatter plots in (a9) and (b9) demonstrate the specific scatter parameters associated with each individual pathological change.

Figure 3.

In (a), histological sections of viable tumor are shown with the extracellular matrix (ECM) segmented and highlighted green. In (b) and (c), the fraction of ECM is shown as a function of μ'_s and γ respectively. In (d), histological sections with low grade tumor, mixed necrosis, and complete necrosis are shown with areas of necrosis shaded green and areas of viable tumor shaded yellow. In (e) and (f), the fraction of necrosis is shown as a function of μ'_s and γ respectively.