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Experimental Recovery of Absorption, Scattering and Fluorescence Parameters in Highly-Scattering Media From a Single Frequency Measurement

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ABSTRACT

In recent years researchers have made significant progress in understanding the physics of fluorescence in highly scattering materials such as tissues in the near-infrared. We have quantitatively verified a model which describes fluorescence in ideal (homogeneous and infinite) tissue-like media. Given the quantitative accuracy of this model, one can use measurements of the fluorescence of a tissue with a homogeneous distribution of fluorophore to obtain the quantum yield, lifetime of the probe, and the absorption and scattering coefficients of the tissue at the fluorescent wavelength. We demonstrate that this can be done with a simple measurement of the photon density as a function of source-detector separation at the excitation and emission wavelengths. To verify our approach we present the lifetime, quantum yield of the fluorescent probe (rhodamine B), and the absorption and scattering coefficients of the medium at the emission peak wavelength that are obtained by a fit of the model to experimental measurements.

Keywords: photon migration, fluorescence, frequency domain, highly scattering media

1. INTRODUCTION

Over the last 10 years, the drive to achieve quantitative near-infrared tissue spectroscopy has led to increased understanding of the behavior of light in tissue-like media. A combination of fluorescence and tissue spectroscopy could prove to be a very useful tool in the detection of diseased tissue and quantitative physiological spectroscopy. Recent progress in the study of photon migration and fluorescence has led to a theory which explains the behavior of fluorescence in a uniform, highly scattering medium [1, 2, 3]. Below we present a brief derivation of this theory followed by experimental measurements in a highly scattering fluorescent medium. Using the theoretical model we fit the data to determine the absorption and scattering coefficients at the emission wavelength, and the lifetime and the quantum yield of the fluorophore. The results of the fit are compared to independent measurements of the parameters.

Propagation of near-infrared light in tissue is frequently modeled using the diffusion approximation to the radiative transfer equation. The fundamental parameters, which describe a tissue that transports light diffusively, are the absorption and scattering coefficients (μ_a and μ_s). The absorption coefficient is one over the mean free path of absorption while the scattering coefficient is one over the mean free path of scattering. Tissues tend to scatter near-infrared light anisotropically, peaked in the forward direction. The anisotropy is characterized by g, the average of the cosine of the scattering angle (usually g~0.8-0.9 in tissues). The reduced scattering coefficient, $\mu_s' = (1-g)\,\mu_s$ is commonly used in place of the actual scattering coefficient because the simplifications of linear transport theory used to arrive at the diffusion equation make g and μ_s inseparable. In a typical tissue, the absorption coefficient in the near-infrared region (accounted for almost completely by hemoglobin and water) is in the range (0.03, 0.20) cm^{-1}. The reduced scattering coefficient, likely due to cellular and subcellular components, is in the range (4.0, 15.0) cm^{-1}.

Fluorescence is a powerful experimental tool because of its high selectivity, sensitivity and potential to provide information about the microscopic environment of the fluorophore. Extrinsic fluorophores can be attached to molecular tagging species yielding a sensitive and specific tool. When adequate rejection of excitation light can be achieved, DC fluorescence intensity can be used to detect small quantities of fluorophore. In addition to DC

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fluorescence intensity, the fluorescence lifetime can be a useful parameter, both for discriminating fluorescence from background and for its use as a parameter that changes depending on local physical conditions. The quantum yield of a fluorescent molecule, which characterizes its efficiency at converting absorbed light to fluorescence emission, also depends on the local molecular environment.

Although there are several standard ways to measure lifetime, the absolute quantum yield of a fluorescent probe can be very difficult to quantify [4]. Measurement of quantum yield traditionally requires a challenging calibration of the detection system and the use of a reference compound. Based on the theory which combines fluorescence and light transport in highly scattering materials, not only can one determine the lifetime of a probe distributed homogeneously in a highly scattering environment, but the quantum yield of the probe can be determined as well. As we show below, this can be done without the use of a reference compound, with simple detection equipment that does not involve a difficult calibration.

2. THEORY

To derive an expression for fluorescence propagation in tissue, we start with the diffusion equation for the emitted light, which is the standard approximation used for strongly scattering media such as tissues:

$$\frac{\partial U}{\partial t} - vD\,\nabla^2 U + \upsilon\mu_a U = q \tag{1}$$

where

$$D = \frac{1}{3\mu_s'} \tag{2}$$

This equation relates the photon density in a turbid medium, U, to the absorption and reduced scattering coefficients of the medium, μ_a and μ_s' respectively, and any sources which may be present, q. ν is the speed of light in the medium. Since, in general, a scattering medium can have different absorption and scattering coefficients at the excitation and emission wavelengths we use subscripts to differentiate which parameters correspond to which wavelength. A subscript m is used to indicate a parameter at the emission wavelength while an x is used for excitation. The frequency-domain Green's function for a point-like source located at the origin and modulated with angular frequency ω is:

$$G(r) = \frac{1}{4\pi\upsilon D}\frac{e^{-k\,r}}{r} \tag{3}$$

where r is the distance from the origin and

$$k^2 = \frac{\mu_a}{D}\left(1 - \frac{i\omega}{\upsilon\mu_a}\right) \tag{4}$$

Consequently, for a source of excitation light at the origin of strength S_x the detected photon density of the excitation light, U_x, is:

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$$U_x = \frac{S_x e^{i\varphi_0}\gamma(\lambda_x)}{4\pi\upsilon D_x}\frac{e^{-k_x r}}{r} \tag{5}$$

where φ_0 is a phase offset due to the experimental setup and $\gamma(\lambda_x)$is the detector efficiency at wavelength λ_x.

The source of emission light is related to the excitation photon density as follows: The probability for exciting the probe is proportional to the excitation photon density, $S_x G_x(r)$. It also depends on the absorption coefficient of the fluorophore for the excitation light, μ_{afx}, which depends linearly on the concentration of the probe. The probability that the excited fluorophore will radiate in a wavelength range $d\lambda_m$ around λ_m is written as $\Lambda\phi(\lambda_m)d\lambda_m$, where Λ is the quantum yield of the fluorophore. When talking about detected signals we also include the spectral response function of the detector, $\gamma(\lambda_m)$. This correction is discussed in detail elsewhere [3].

There is a temporal delay in the emission of the fluorescence photon due to the fluorophore's finite lifetime, τ. This delay can be accounted for by convoluting the exponential lifetime decay with the actual time the excitation photon is absorbed. All of this is expressed in the differential frequency-domain emission source term below:

$$dq_m = \upsilon\mu_{afx} S_x G_x(r)\left(\frac{1+i\omega\tau}{1+\omega^2\tau^2}\right)\Lambda\phi(\lambda'_m)d\lambda'_m \tag{6}$$

The detected photon density of the emission light is found by convoluting the Green's function at the emission wavelength with the source distribution above and integrating $d\lambda'_m$ over the detector bandwidth:

$$\begin{aligned} U_m &= \int G_m(r) * dq_m \\ &= \left(\frac{S_x e^{i\varphi_0}\gamma(\lambda_x)\mu_{afx}\Lambda\int\phi(\lambda'_m)\dfrac{\gamma(\lambda'_m)}{\gamma(\lambda_x)}d\lambda'_m}{4\pi\upsilon}\right)\left(\frac{1+i\omega\tau}{1+\omega^2\tau^2}\right)\left[\left(\frac{1}{(k_m^2-k_x^2)D_m D_x}\right)\frac{e^{-k_x r}-e^{-k_m r}}{r}\right] \end{aligned} \tag{7}$$

where the ratio $\dfrac{\gamma(\lambda'_m)}{\gamma(\lambda_x)}$ is a measurable parameter of the experimental setup that accounts for the spectral response of the detector. Equation (7) can be used to calculate the primary fluorescence at a given location.

At this point we note that Eq. (7) is the product of three factors. The first term has contains information about the fluorophore concentration (in μ_{afx}) and quantum yield (Λ). The second term has the lifetime contribution, which has the effect of adding a phase delay to the signal. The third term depends on the absorption and reduced scattering coefficients of the medium at the excitation and emission wavelengths. Note that the concentration of the fluorophore does influence the absorption coefficient at both the excitation and emission wavelength, depending on the spectral overlap of the absorption and emission spectra. The simplicity of this equation is a result of our choice to work in the frequency-domain. The Fourier Transform of this equation into the time-domain is complicated and does not yield an explicit function [5].

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3. EXPERIMENT

We present results from a multi-distance single-frequency measurement of fluorescence emission in the infinite medium geometry. Measurements were made on a highly scattering liquid phantom with rhodamine B added as a fluorophore. The absorption and reduced scattering coefficients of the medium at the excitation wavelength (532 nm) (See Table 1) were determined using the multi-distance frequency-domain protocol[6].

Table 1 : Optical coefficients at the excitation wavelength

Parameter	λ=532 nm
μ_{ax} (cm^{-1})	0.124 ± 0.003
μ_{sx}' (cm^{-1})	10.9 ± 0.3

Experimental data for fit

The experimental data set used in the fit consists of measured DC intensity and phase of the fluorescence emission photon density wave as a function of source-detector separation. DC intensity and phase were recorded over a separation range from 1 to 2.6 cm. The phase offset due to the experimental setup has been subtracted from the measured phases. We calculated the product $S_x\gamma(\lambda_x)$ from Eq. (5), using the measurements made to calculate the absorption and reduced scattering coefficients at the excitation wavelength.

Measurements were made using the frequency-domain spectrometer schematically shown in Figure 1. The

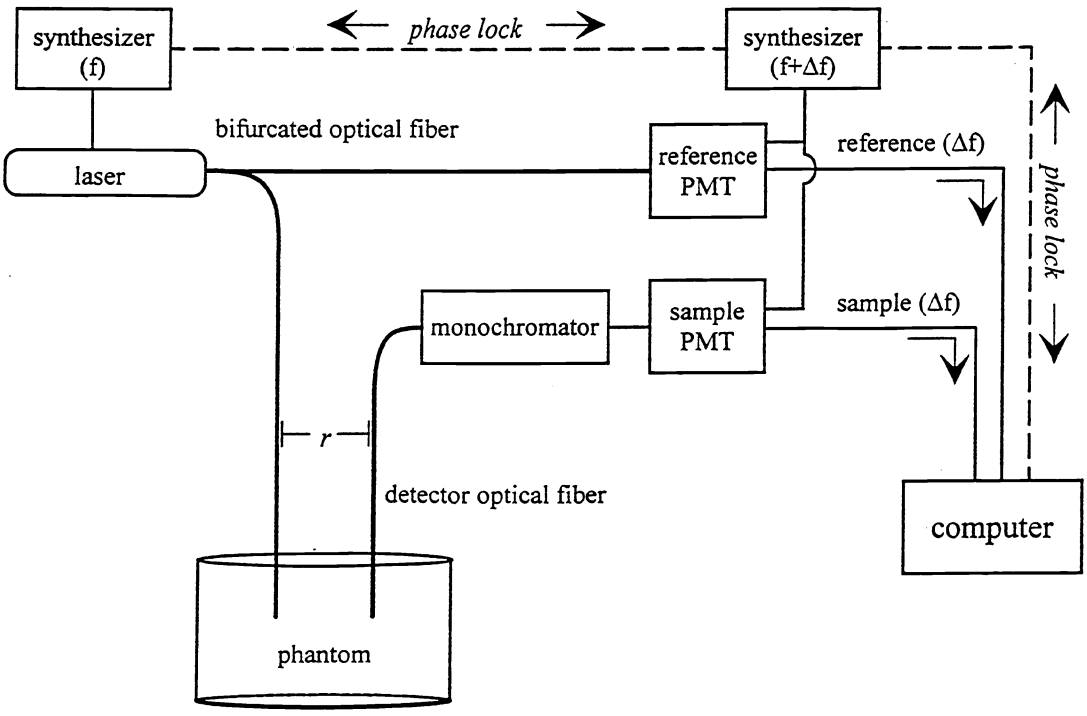

Figure 1. Schematic of frequency-domain spectrometer

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details of operation of this instrument are discussed elsewhere [7]. Briefly, a pulsed frequency-doubled Nd:YAG laser is used to generate a pulse of light at 532 nm at a repetition rate of 76.2 MHz. One arm of a bifurcated fiber optic carries most of the light to the scattering sample. After some distance in the sample, light is collected with another fiber optic cable which carries it to a monochromator, used to reject excitation light. After the monochromator, the light is detected using a heterodyned photomultiplier tube (PMT) which down converts the phase information in the 76.2 MHz component of the optical signal to an electrical signal at 400 Hz. This signal is digitized, averaged and Fourier transformed to yield the AC, DC and Phase of the photon density wave. Corrections for fluctuation in the laser are made using the light from the second part of the bifurcated fiber used to transport the light from the laser to the medium.

The absorption and reduced scattering coefficients at the emission wavelength (580 nm) were also determined using the standard multi-distance protocol[6]. For this measurement we used a dye-laser tuned to 580 nm and the same detection and collection system. The lifetime of the rhodamine B in water was measured to be 1.50 ± 0.01 ns in our laboratory. The quantum yield of rhodamine B in water is reported to be 0.31 [8]. We assume an error of 10% for this value based on consideration of standard methods used to measure quantum yield[4].

The fitting procedure

The coefficients of the medium at the excitation wavelength (μ_{ax}, μ_{sx}') were independently determined as indicated above. To obtain the absorption and reduced scattering coefficients of the medium at the emission wavelength, (μ_{am}, μ_{sm}'), and the lifetime (τ) and quantum yield (Λ), we fit the measured phase and DC of the fluorescence emission on the basis of Eq. (7). The global reduced χ^2 was minimized by varying these four parameters, (μ_{am}, μ_{sm}', τ, Λ), yielding a reduced χ^2 of 1.03. Minimization and rigorous error analysis was performed using the GLOBALS Unlimited software package (Laboratory for Fluorescence Dynamics, Dept. of Physics, University of Illinois at Urbana-Champaign) [9].

4. RESULTS

Table 2 indicates the independently measured parameters and the same parameters derived from the fitting process described above.

Table 2.

Parameter	independent measurement	fit
μ_{am} (cm^{-1})	0.087 ± 0.004	0.076 ± 0.002
μ_{sm}' (cm^{-1})	10.1 ± 0.3	10.9 ± 0.5
τ (ns)	1.50 ± 0.01	1.45 ± 0.04
Λ	0.31 ± 0.03	0.35 ± 0.02

Figures 2 and 3 show the experimental data (symbols) and the fit using Eq. (7) (lines). Experimental errors are smaller than the markers in both plots.

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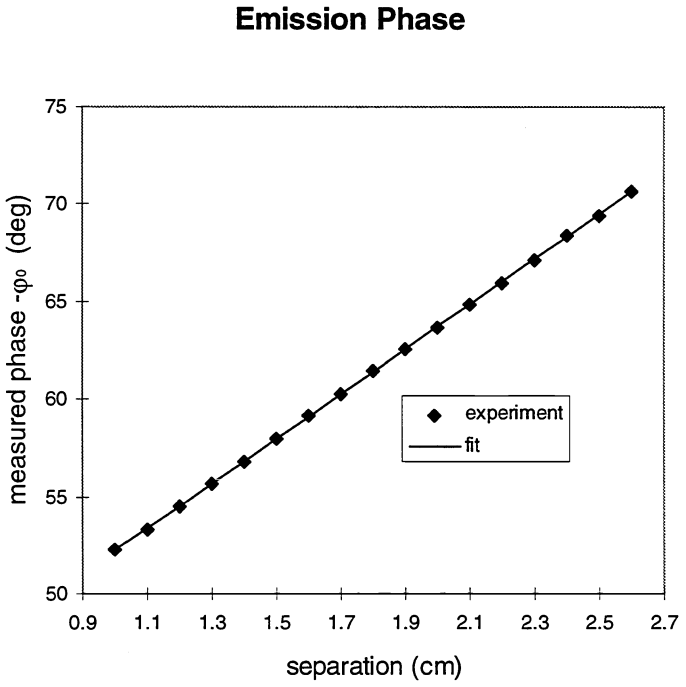

Figure 2. Plot of the experimental phase data along with the fit.

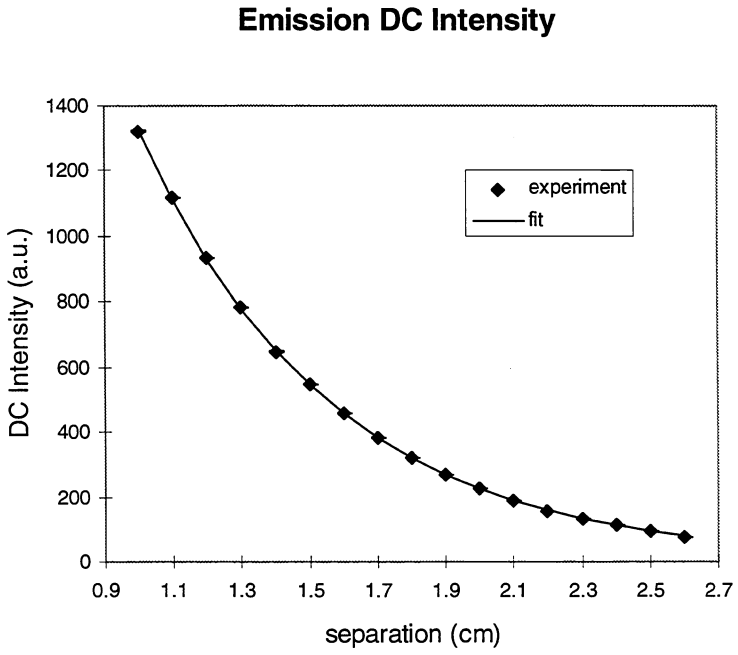

Figure 3. Plot of the experimental DC intensity data along with the fit..

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5. DISCUSSION

The fitted parameters are in good agreement with the independently determined values eventhough they are not strictly within the errors. We are investigating several potential causes of the deviations. For instance, one possible cause of the discrepancy is, an inappropriate independent determination of the absorption and scattering coefficients at the emission wavelength. There is an important difference between the independent measurement of μ_{am} and μ_{sm}' and the measurement of the fluorescence emission. In the independent measurement of μ_{am} and μ_{sm}' we have collected essentially only photons at λ_m. On the other hand, in the fluorescence measurement the detected photons cover the whole detector bandwidth. Thus, the measured emission photons experience a range of absorption and reduced scattering coefficients. Therefore, strictly speaking, the two experiments do not measure exactly the same parameters. Estimation of this effect on the measured parameters is complicated by the structure of the emission spectrum of the probe, the absorption spectrum of the medium and the details of the detector response as a function of wavelength.

Regardless of the small discrepancy, the method does provide the quantum yield and lifetime of the fluorophore within rather small errors. Note that this measurement is done without the use of a reference compound and therefore is an absolute determination. Such accurate absolute determination of the quantum yield has application both in the basic study of fluorescent molecules and in tissue spectroscopy. Our experiment not only indicates a potentially new method of determining the quantum yield, but it also offers a method for finding the concentration of a fluorophore with known quantum yield homogeneously distributed in a highly scattering material.

In a real physiological tissue sample one does not have the luxury of working with an infinite homogeneous sample. However, the adaptation of the theory to semi-infinite boundary conditions is straight forward and the same procedure should be applicable.

6. CONCLUSION AND FUTURE DIRECTIONS

We have presented a new method to measure the absolute quantum yield and lifetime of a fluorescent probe uniformly distributed in a highly scattering medium. Simultaneously the optical coefficients of the medium at the emission wavelength are also recovered. We are in the process of evaluating the multi-distance protocol in the presence of a fluorophore. We are continuing investigation in the following areas: the effects of secondary fluorescence, the case of multiple fluorophores, the application of this technique to the semi-infinite geometry, and the case of inhomogeneous distribution of fluorophores.

7. ACKNOWLEDGEMENTS

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