Quantitative Fluorescence Spectroscopy
in Strongly Scattering Media Containing Multiple Fluorophores

Albert Cerussi, Sergio Fantini, and Enrico Gratton

Laboratory for Fluorescence Dynamics
University of Illinois at Urbana-Champaign
Department of Physics
1110 West Green Street, Urbana, IL 61801-3080

ABSTRACT

We have used diffusion theory to model the fluorescence photon density inside a strongly scattering medium containing multiple fluorophores. Frequency-domain parameters were measured as functions of source-detector separation and modulation frequency inside a turbid medium containing two fluorophores. Our theoretical prediction, based on a model originally proposed by Patterson and Pogue, was found to be in excellent agreement with the experimental data.

KEY WORDS

Fluorescence Spectroscopy, Fluorescence Lifetime, Tissue Spectroscopy, Diffusion Theory

OCIS CODES

170.5280, 170.6280, 290.4210, 300.6280

I. INTRODUCTION

A. NEAR-INFRARED TISSUE SPECTROSCOPY

A heroic goal of tissue spectroscopy is to explore non- or minimally-invasively the physiological state of a tissue. Photons within the spectral region of 700-900 nm can penetrate several cm into tissue. Hemoglobin provides excellent optical contrast in the 700-900 nm region since there it is the dominant absorber. Unfortunately, other compounds of physiological importance in tissues show little if any absorption in this same spectral region. In addition, local environment variables (such as pH) play an important physiological role and are not easily monitored in this spectral region. Poor native contrast may be overcome by using exogenous contrast agents.

B. FLUORESCENCE LIFETIME SPECTROSCOPY

The most notable strengths of fluorescence spectroscopy are highly specific staining (for example, using antibodies) and enhanced sensitivity, especially in the low chromophore concentration regime. There are a variety of emission parameters that depend upon the fluorophore local environment, such as the emission spectrum, the emission lifetime, and the emission quantum yield. The fluorescence lifetime in particular provides a quantitative spectroscopic parameter that is sensitive to the fluorophore local environment. Many fluorophores exist that exhibit measurable lifetime changes while in the presence of physiologically relevant substances such as Ca++ and Mg++, or as a consequence of variations in local environment conditions such as pH. Research into the fabrication of such fluorophores for in vivo use is in progress [1].

C. THE PROBLEM OF MULTIPLE SCATTERING

The stumbling block for optical tissue spectroscopy is strong multiple scattering. Patterson and Pogue derived an analytic expression for the emission photon density for the case of a fluorophore distributed uniformly throughout a multiple-scattering medium [2]. The fluorophore properties, namely the quantum yields, the lifetime, and the emission and absorption spectra, have all been successfully recovered from within a multiple-scattering tissue phantom [3]. This diffusion-based model is the cornerstone of our work. Others have used this equation for imaging purposes [4-6].

D. PURPOSE OF STUDY

The long-term goal of this work is to apply fluorescence spectroscopic techniques to tissues. We have extended the model of Patterson and Pogue to the more general case of N fluorophores homogeneously
dispersed inside of a multiple-scattering medium. This paper will emphasize the recovery of fluorescence lifetimes of multiple fluorophores from within a multiple-scattering medium.

II. THEORY

The diffusion equation relates the photon density, \( U(r,t) \) (photons/cm\(^{-3}\)) to the optical coefficients of the medium; namely the absorption coefficient \( \mu_a \) (cm\(^{-1}\)) and the reduced scattering coefficient \( \mu'_s \) (cm\(^{-1}\)) [7]. Both excitation and emission photon transport are well described as diffusive processes in homogeneous multiple-scattering media, although these transport equations have different source terms.

A. NOTATION CONVENTIONS

Our chromophores may be divided into two groups. The background chromophores, designated by the subscript \( b \), form the first group. In this treatise, the term ‘background’ is employed to mean anything that is not fluorescent. The \( N \) independent fluorescent chromophores, designated by the subscript \( f \), form the second group. Further, we shall use the subscript \( n \) as a species index for the fluorophores. Thus, the total absorption of the medium takes the form:

\[
\mu_{a,m} = \mu_{a,b} + \sum_{n=1}^{N} \mu_{a,f_n}.
\]

\[
\mu_{s,m} = \mu_{s,b} + \sum_{n=1}^{N} \mu_{s,f_n}.
\]

We have also used the subscript convention of \( x \) and \( m \) to denote the excitation and the emission wavelengths, respectively. Thus, \( \mu_{a,m} \) represents the absorption of the medium as a whole at the emission wavelength (i.e., \( \lambda_m \)), whereas \( \mu_{a,f_n} \) represents the absorption of only the fluorescent species \( n \) at \( \lambda_m \).

B. EXCITATION PHOTON DENSITY

The excitation source term is assumed to be an isotropic point source. The solution to the diffusion equation with infinite medium boundary conditions for a point source that is intensity-modulated at frequency \( \omega/2\pi \) yields the familiar solution of the form \( U_i(r,\omega)\exp(-i\omega t) \) [8]. If we define \( P_\delta(\omega) \) as the source strength (photons/s\(^{-1}\)), \( \phi_s \) as the source phase (degrees) \( v \) as the speed of light in water, \( \nu D_e \equiv \nu(3\mu_a)^{-1} \) as the excitation diffusion coefficient (cm\(^2\)/s\(^{-1}\)), then the excitation photon density \( U_i(r,\omega) \) has the form:

\[
U_i(r,\omega) = \frac{P_\delta(\omega)}{4\pi\nu D_e r} \exp[-k_i(\omega)r] \exp[-i\phi_s],
\]

where \( k_i(\omega) \) is the photon density wave-vector (cm\(^{-1}\)):

\[
k_i^2(\omega) = \frac{\mu_a}{D_e} \left( 1 - \frac{i\omega}{\nu\mu_a} \right).
\]

C. EMISSION PHOTON DENSITY

While the excitation is constrained to a point source, the emission source is a distribution of point sources dispersed throughout the entire medium. Any fluorophore may become a point source after absorbing an excitation photon. Details on the form of the emission source may be found elsewhere [3]. If we add up the photon density waves from all \( N \) fluorescent species, and include the detector response, we obtain the total detected emission photon density:

\[
U_m(r,\omega) = \sum_{n=1}^{N} \left( \int dU_n(r,\omega) \right)
\]

\[
= \sum_{n=1}^{N} \left( \frac{1 + i\omega r_n}{1 + (\omega r_n)^2} \Lambda_n \mu_{e,f_n} \Phi_n \right) 
\times \frac{1}{r} \exp\left[ -k_n^2(\omega) r \right] \exp\left[ -k_n(\omega) r \right]
\times \frac{P_n(\omega)}{4\pi\nu D_e D_m} \exp[-i\phi_e].
\]

Equation (4) assumes no interactions between fluorescent species as well as negligible photobleaching. It further assumes a negligible contribution from fluorescence light that is re-absorbed and subsequently re-emitted; once an emission photon is absorbed, it is assumed that it cannot be re-emitted. \( \Phi_n \) is defined as the probability that our detector will detect an emission photon from species \( n \) (details may be found in Ref. [3]). \( \Phi_n \) depends upon the detector wavelength response, the detector spectral bandpass, and also the emission probability of species \( n \). The emission optical coefficients now reflect average values taken over the spectral bandpass of the detector.

III. EXPERIMENT

A. TISSUE PHANTOM: BACKGROUND

In order to simulate the scattering and absorption properties of tissues exposed to near-infrared light, we constructed a tissue phantom comprised of water, Liposyn III (20% solids, Abbott Laboratories, Chicago, IL), and black India ink. We prepared this sample with 8200 mL of water, pH buffered at 9.0, and then added 300 mL of the highly scattering Liposyn and a tiny
amount of black India ink. This portion of the sample comprises the background.

B. TISSUE PHANTOM: EXOGENOUS FLUOROPHORES
Two fluorophores were used in this experiment: 5&6 carboxyfluorescein (mixed isomers: Molecular Probes, Eugene, OR) and fluorescein (reference standard: Molecular Probes). Both absorption and emission spectra, measured in standard equipment, are presented in Figure 1. Absorption and emission spectral information is shown in Table I [9]. The manufacturer provided the peak spectral values, and we measured the extinction values at 488 and 540 nm using the normalized spectra in Figure 1. We measured the carboxyfluorescein lifetime on a frequency-domain lifetime instrument. The quantum yield for carboxyfluorescein was measured relative to fluorescein in a fluorometer, and yielded a value consistent with that of eosin [10]. All samples were buffered at pH 9 with a 25 mM TRIS solution. The final fluorophore concentrations in the phantom were 258 nM (carboxyfluorescein) and 108 nM (fluorescein).

These two fluorophores were chosen because they are easily soluble in water, pH insensitive (for high pH), and inexpensive. We observed no significant photobleaching of our sample, and the emission decays of both fluorophores are very well described with a single decay rate. Thus, both of these fluorophores are well in line with our assumptions. Furthermore, they possess different lifetimes, which may be a contrast enhancement in tissues.

C. INSTRUMENTATION
The basic components of the frequency-domain instrument are presented in Figure 2. The 488 nm line of an Argon-Ion laser provided the excitation. An Acousto-Optical Modulator (AOM) provided the intensity modulation (IntraAction Corp., Bellwood, IL); this AOM was driven by a radio-frequency sine wave of frequency $f$ that was amplified to a final power of 1 W. A heat bath held the AOM crystal at a constant temperature to avoid drifts in the modulation depth. The modulated light was collected by a 10X microscope objective and then focused down into a 2 mm core diameter fiber. Some light was taken for an optical reference channel by attaching directly a smaller 1 mm core diameter fiber to the side of the larger fiber.

The larger core fiber was placed inside a cylindrical tank containing the 8.5 L fluorescent scattering suspension. A three-axis positioning device (not shown) moved this fiber anywhere inside the sample. A 0.6 mm core diameter detector fiber was placed at a distance $r$ away. This fiber was placed well inside the medium, so that we simulated an infinite medium geometry. We used an aspheric lens and a bi-concave lens to collimate the collected light. This collimation is necessary for the interference filters that follow since the throughput of these filters depends heavily upon the angle of the light striking the filter.

When studying the excitation signal, we used an interference bandpass filter centered at 488 nm with a 10 nm FWHM (F10-488.0-4-1.00, CVI, Putnam, CT). When studying the emission, we used a combination of three filters: an interference bandpass filter centered at 540 nm with a 10 nm FWHM (F10-540.0-4-1.00 CVI), plus two 515 nm longpass filters (CG-OG-515-1.00, CVI). The total transmission of this filter combination is approximately 30% at 540 nm, and provides at least 7 OD rejection at 488 nm.

The collected light was then focused onto a photomultiplier tube (PMT). This sample channel PMT
was driven by another amplified synthesizer signal, this time at a frequency of $f=0.00125$ MHz. A heterodyning effect was achieved, which produced a 1250 Hz signal that was digitized, Fourier transformed, and analyzed with a personal computer [11]. A master synthesizer provided both a 2 MHz clock signal for the data-acquisition card, and a 10 MHz reference signal to phase lock the synthesizers and the data acquisition.

D. EXPERIMENTAL PROCEDURE

After preparing the sample, we measured the optical coefficients of the medium at $\lambda$ using a multi-distance protocol as described by Fantini et al. [12]. We calculated the emission optical coefficients based upon the known spectra of the medium. We then averaged the emission optical coefficients over the detector bandpass centered at 540 nm to get $\mu_{em}$ and $\mu_{cm}$. The optical coefficients are presented in Table II.

After placing the excitation-pass filter in the detector, we performed a multi-distance measurement by moving the source fiber. This measurement allowed us to determine the source terms $P_s(0)$ and $\phi$. We then switched to the three emission-pass filters, and performed another multi-distance measurement, but this time collecting emission photons. Measurements were performed at eight different source modulation frequencies, ranging from 72.46 to 199.12 MHz. Since the source terms vary with modulation frequency, we repeated both excitation and emission measurements for each of the eight source-modulation frequencies.

<table>
<thead>
<tr>
<th>OPTICAL COEFFICIENT</th>
<th>EXCITATION ($\lambda_e=488$ nm)</th>
<th>EMISSION ($\lambda_m=540$ nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>background absorption (cm$^{-1}$)</td>
<td>0.0465(5)</td>
<td>0.0422(5)</td>
</tr>
<tr>
<td>total absorption (cm$^{-1}$)</td>
<td>0.0897(7)</td>
<td>0.0529(6)</td>
</tr>
<tr>
<td>reduced scattering (cm$^{-1}$)</td>
<td>12.7(1)</td>
<td>10.5(8)</td>
</tr>
</tbody>
</table>

IV. RESULTS

In the graphs that follow it is important to note that we did not fit Eq. (4) to the measured data. Equation (4) has many variables that may compensate for one another, so we elected to make predictions based on the known parameters of the medium.

A. MULTIPLE-DISTANCE MEASUREMENT

Figure 3 provides the experimental measurement (points) versus the prediction of Eq. (4) (solid lines) over a range of source-detector separations with a source modulation frequency of 72.46 MHz. This prediction used the values of the parameters given in Tables I and II. Both the prediction for the AC amplitude (bottom set of Figure 3a) and the DC intensity (top set of Figure 3a) show excellent agreement with the measurement. It is important to note that the source terms and the detector calibration factor are not floating parameters; rather, they have been determined a priori as described above. We have also drawn confidence intervals (broken lines) which represent the compound errors in each of the parameters used in our predictions. Errors in the optical coefficients affect the slopes of these lines. Errors in $\Lambda$ and $\tau$ have varied effects. The AC intensity is a phasor sum of both fluorescein and carboxyxy eosin photon density waves. Thus, the relative weight for each species is affected by both $\Delta \mu_{def}$ and $\tau$. The DC intensity, which is a linear sum, is unaffected by $\tau$, yet the DC intensity for each species is weighted by the factor $\Delta \mu_{def}$.

The measured emission phase in Figure 3b also shows good agreement with the prediction, but not as good as for the AC and the DC. As it turns out, Liposyn is actually more realistic of a tissue phantom than we thought, since it auto-fluoresces when excited...
at 488 nm. Although the magnitude of the auto-fluorescence intensity is only about 1% of the detected AC signal, this tiny contribution has a non-negligible effect upon the phase. The same errors that affect the AC intensity will affect the phase, although the effect is clearly more pronounced for the phase. The large confidence interval is mostly due to uncertainties (i.e., the auto-fluorescence) in $\phi$.

**B. MULTIPLE-FREQUENCY MEASUREMENT**

We also present in Figure 4 the modulation frequency dependence of the emission AC amplitude (left dependent axis) and the emission phase (right dependent axis). Once again, the symbols represent the measured values, and the lines represent the predictions of Eq. (4) using the values of Tables I and II. The agreement is again very good. Unlike the case of systematic error in Figure 3a, the points appear both above and below the prediction. This stagger is especially true of the phase, where the error in $\phi$ varies from frequency to frequency.

**V. DISCUSSION**

Equation (4) quantitatively reproduced the measured experimental data. This is expected, since the single fluorophore equations may be added together. However, since the end result is a phasor, both amplitude (AC intensity) and phase (which includes both lifetime and photon diffusion) are relevant.

The case of auto-fluorescence poses a problem. In principle, one could fit for the effective lifetime and the quantum yield of this background fluorescence. Auto-fluorescence may have a large effect upon the final emission signal. Thankfully, tissue auto-fluorescence weakens with increasing excitation wavelength. Therefore, when using fluorophores such as indocyanine green (a FDA approved fluorophore that absorbs strongly at 780 nm) tissue auto-fluorescence becomes small.

Just because the auto-fluorescence is small, however in no way implies that it’s effect is negligible. Errors in the phase may be significant if the auto-fluorescence lifetime(s) is (are) very different form the lifetimes of the fluorophores of interest. In cases where this is true, one can resort to using just the intensities to find the lifetime. The DC intensity may be used to find the relative fractions of each component (i.e., $\Lambda_{\text{D}} \mu_{\text{A}}$). The AC intensity could subsequently be used to determine the lifetimes of each of these components.

Clearly, fitting would be more appropriate in a real-life situation. Fitting Eq. (4) is tricky because the different parameters compensate for one another. For this reason, a global fit is appropriate, for example fitting the AC intensity simultaneously with the phase.
We have taken this approach for the single fluorophore case with excellent results [13], and we will modify the routine to include multiple fluorophores.

VI. CONCLUSION

The original model of Patterson and Pogue has been extended to the general case of N fluorophores. This model quantitatively describes the generation and propagation of fluorescence inside of homogeneous multiple-scattering media. When the source terms are properly taken into account, one can quantitatively interpret data measured with multi-distance and/or multi-frequency approaches.

VII. ACKNOWLEDGMENTS

This work was performed at the Laboratory for Fluorescence Dynamics in Urbana, IL, and was supported by the National Institutes of Health (grants RR03155 and CA57032) and the University of Illinois.

VIII. REFERENCES AND NOTES


[9] Molecular Probes provided the spectral data parameters for the sample lot number, but did not provide measurement errors for those parameters.


