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Relation between fluorescence spectra of dilute and turbid samples

A. J. Durkin, S. Jaikumar, N. Ramanujam, and R. Richards-Kortum

We present a method to extend rank-annihilation-factor analysis (RAFA) for the analysis of fluorescence from homogeneous turbid samples. The method is based on a fundamental relationship between the fluorescence of a dilute solution and that of a turbid solution. We have derived this relationship, known as the transfer function, for turbid materials using the two-flux Kubelka–Munk theory. The method is tested with spectroscopic data from optically thin and turbid samples of the media of a human aorta. At 450-nm excitation, agreement between the measured and predicted dilute-solution fluorescence spectra is within 5% at all emission wavelengths; at 340-nm excitation, agreement is within 20% at all wavelengths, with some residual Soret-band absorption. The simulations presented indicate that the transfer function is markedly more sensitive to absorption than to scattering properties.

Key words: Fluorescence, scattering, turbid, Kubelka–Munk theory, tissue.

Introduction

Advances in fiber-optic, light-source, and detector technology have made it possible to remotely monitor the diffuse reflectance and fluorescence spectra of human tissue *in vivo* in real time.^{1,2} Spectroscopic methods have recently been explored as a means of studying pathology in human tissue.^{1–7} However, deconvolution of tissue spectra to yield the concentration of tissue components of interest has proven difficult for two reasons: (1) human tissue is a highly scattering and a highly absorbing medium,⁸ and (2) the spectra of many biological chromophores overlap.^{6,9}

Several models of light propagation in tissue have been proposed to deconvolve the effects of absorption and scattering from tissue reflectance and transmission, and several authors have attempted to use simple models to describe tissue-fluorescence spectra in terms of the tissue's optical properties.^{3,7,10} Typically, chromophore concentrations are determined by fitting the tissue spectra to a mathematical description using least-squares techniques, where the chromophores' optical properties are known and the concentrations are used as the free parameters of the fit. Although the least-squares methods enable de-

convolution of the contributions from various tissue chromophores, these methods require knowledge of the number of chromophores present and of the optical properties of each.^{3,7} Often this information is unavailable.

Techniques have recently been developed to quantify the concentration of fluorescing knowns in the presence of unknown fluorophores in dilute solutions.^{11–14} These techniques, known as rank-annihilation-factor analysis (RAFA), are based on analysis of fluorescence excitation–emission matrices (EEM's). A concise review¹⁵ of the RAFA method is included in the appendix. The RAFA method has great potential for improving the analysis of tissue-fluorescence data where the sample composition is unknown and variable. Unfortunately, the application of RAFA is predicated on having a sample that can be described optically as a dilute solution; these methods have not yet been successfully applied to samples that significantly scatter or absorb light.

In this paper, we present a method for modeling tissue fluorescence that relates the fluorescence EEM of a turbid sample to the effective dilute-solution EEM, where the effects of scattering and reabsorption have been deconvolved. We evaluate the transfer function using the two-flux Kubelka–Munk theory for turbid samples. Furthermore, we show that the transfer function can be determined from measurements of diffuse reflectance and transmittance in turbid samples. We test this model using data from optically thin and turbid samples of the media of a human aorta. Finally, we illustrate the behavior of the transfer function for a number of simulations in

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which the absorption and scattering coefficients are varied over values typical for human tissue in the UV and visible regions of the spectrum.

Theory

The fluorescence EEM of a dilute solution with N fluorophores is a matrix that contains the detected fraction of the total fluorescence intensity $S_{\text{Total}}(\lambda_i, \lambda_j)(\Omega/4\pi)$, normalized to the excitation intensity $I(\lambda_i)$ and the sample thickness L_{dil} . The fraction of fluorescence detected is given by Ω , the solid angle of collection of the detector, over 4π the total solid angle assuming isotropic emission of fluorescence. The ij th element of this matrix is given by

$$\begin{aligned} \text{EEM}_{\text{Dil},ij} &= \left(\frac{\Omega}{4\pi} \right) \frac{S_{\text{Total}}(\lambda_i, \lambda_j)}{I(\lambda_i)L_{\text{dil}}} \\ &= \left(\frac{\Omega}{4\pi} \right) \sum_{k=1}^N 2.3C_k\epsilon_k(\lambda_i)\phi_k(\lambda_j), \\ &= \left(\frac{\Omega}{4\pi} \right) \sum_{k=1}^N \mu_{a_k}(\lambda_i)\phi_k(\lambda_j), \end{aligned} \quad (1)$$

where each fluorophore can be characterized by a concentration C_k , a wavelength-dependent molar-absorption coefficient $\epsilon_k(\lambda_i)$, and a wavelength-dependent fluorescence quantum yield, $\phi_k(\lambda_j)$.⁹ In writing Eq. (1), we have assumed that the sample is dilute and that the fluorescence quantum yield depends only on the emission wavelength.⁹ The first assumption is valid for nonscattering samples where the sum of the optical densities at the excitation and emission wavelengths is less than 0.1.⁹ The second assumption is valid for cases in which energy transfer is negligible and fluorescence occurs only from the first excited electronic-energy level. This is generally true for molecules in condensed phases, with a few rare exceptions.⁹

Model for Turbid-Sample Fluorescence

Because human tissue is highly scattering in the UV and visible regions of the spectrum, it cannot be described as a dilute solution. In particular, it has been shown that scattering and reabsorption significantly affect the fluorescence spectra of tissues in the visible and UV regions of the spectrum.^{3,6,10,16} In general, a one-dimensional model of fluorescence must describe the attenuation of the excitation beam as it passes through the turbid sample and the reattenuation of the fluorescence as it travels from the point of generation to the surface of the sample. These effects can be described mathematically by recognizing that a turbid sample can be treated as a dilute solution if its thickness is small compared to the photon mean free path.³ Dividing a turbid sample of thickness L_{Tur} into a number of thin layers of thickness dz along the optical axis allows the contribution to the front-surface fluorescence spectrum of a thin layer of tissue of thickness dz , located at depth z ,

to be calculated as

$$\begin{aligned} dS_{\text{TurFront}}(\lambda_i, \lambda_j, z) &= I(\lambda_i)H_{\text{in}}[\text{op.prop.}(\lambda_i), z, L_{\text{Tur}}] \\ &\times \left[\sum_{k=1}^N 2.3C_k\epsilon_k(\lambda_i)\phi_k(\lambda_j)dz \right] \\ &\times H_{\text{out}}[\text{op.prop.}(\lambda_j), z, L_{\text{Tur}}], \end{aligned} \quad (2)$$

where H_{in} describes the fraction of the incident excitation light that reaches the tissue layer of concern, and H_{out} describes the fraction of fluorescence generated at z that reaches the front of the tissue surface. This is illustrated for diffuse illumination in Fig. 1.

The total fluorescence emitted from the front surface of a sample of thickness L_{Tur} is given by integrating over the turbid sample:

$$\begin{aligned} S_{\text{TurFront}}(\lambda_i, \lambda_j) &= \int_0^{L_{\text{Tur}}} dS_{\text{TurFront}}(\lambda_i, \lambda_j, z) \\ &= I(\lambda_i) \int_0^{L_{\text{Tur}}} dz H_{\text{in}}[\text{op.prop.}(\lambda_i), z, L_{\text{Tur}}] \\ &\times \left[\sum_{k=1}^N 2.3C_k\epsilon_k(\lambda_i)\phi_k(\lambda_j) \right] \\ &\times H_{\text{out}}[\text{op.prop.}(\lambda_j), z, L_{\text{Tur}}]. \end{aligned} \quad (3)$$

If fluorophores are distributed homogeneously within the sample, the summation can be taken outside the integral, yielding:

$$\begin{aligned} S_{\text{TurFront}}(\lambda_j, \lambda_j) &= I(\lambda_i) \left[\sum_{k=1}^N 2.3C_k\epsilon_k(\lambda_i)\phi_k(\lambda_j) \right] \\ &\times \int_0^{L_{\text{Tur}}} dz H_{\text{in}}[\text{op.prop.}(\lambda_i), z, L_{\text{Tur}}] \\ &\times H_{\text{out}}[\text{op.prop.}(\lambda_j), z, L_{\text{Tur}}]. \end{aligned} \quad (4)$$

Fig. 1. Geometry for the fluorescence model. H_{in} represents the probability that an excitation photon (solid arrows) will reach a thin layer dz , located at depth z below the sample surface. Similarly, H_{out} represents the probability that a fluorescence photon (dashed arrows) generated in the layer dz will reach the front surface of the sample.

We define the ij th element of EEM_{Tur} to be the detected fraction of the fluorescence emitted from the sample front surface, normalized to the incident intensity and sample thickness:

$$EEM_{Tur,ij} = \frac{S_{TurFront}(\lambda_i, \lambda_j, L_{Tur})}{I(\lambda_i)L_{Tur}} \left(\frac{\Omega}{2\pi} \right). \quad (5)$$

Note that the detected fraction of fluorescence emitted from the sample *front* surface is given by $\Omega/2\pi$. Combining Eqs. (1), (4), and (5) yields

$$\begin{aligned} EEM_{Tur,ij} &= \frac{2EEM_{Dil,ij}}{L_{Tur}} \int_0^{L_{Tur}} dz H_{in}[\text{op.prop.}(\lambda_i), z, L_{Tur}] \\ &\quad \times H_{out}[\text{op.prop.}(\lambda_j), z, L_{Tur}] \\ &= EEM_{Dil,ij} \frac{2TF[\text{op.prop.}(\lambda_i, \lambda_j), z, L_{Tur}]}{L_{Tur}}. \end{aligned} \quad (6)$$

Thus the turbid EEM is related to the EEM of the equivalent dilute solution by the wavelength-dependent transfer function TF.

The form of the transfer function depends on the details of light propagation within the sample. Several models of light propagation have been described in the literature and can be used to calculate the dependence of the transfer function on the sample's optical properties, collection and detection geometry, and mismatch in the index of refraction at the tissue surfaces. Once the form of the transfer function has been determined, its value can be computed as a function of the excitation and emission wavelengths, given the total optical properties of the turbid sample at these wavelengths and the thickness of the sample. Depending on the model of light propagation that is assumed and on the geometry of the excitation source with respect to the sample surface, the total optical properties can be determined by measurements of diffuse reflectance and transmission.^{17,18} Sample thickness can be determined from physical measurements. Equation (6) can then be used to calculate the equivalent-dilute-solution EEM, and conventional RAFA techniques can subsequently be applied to analyze this equivalent-dilute EEM.

In the next section of the paper, we evaluate the form of the transfer function using the two-flux Kubelka–Munk theory for turbid samples. We have assumed diffuse illumination and no index-of-refraction mismatch between media. Despite this simplifying assumption and because fluorescence is an isotropic process to a first approximation, mismatches in the index of refraction will affect the EEM of both dilute and turbid samples in the same way.

Derivation of the Transfer Function

In Kubelka–Munk theory, diffuse-light flux in a scattering medium is divided into two parts, one traveling in the $+z$ direction, the other in the $-z$ direction, assuming the geometry of Fig. 1. With these assumptions, the diffuse intensity reaching a depth z , and thus H_{in} , can be calculated in terms of the Kubelka–

Munk absorption A_{KM} and scattering coefficients S_{KM} as

$$H_{in}(\lambda_i, z, L_{Tur}) = 2C(\lambda_i, L_{Tur})e^{\kappa_i z} + 2B(\lambda_i, L_{Tur})e^{-\kappa_i z}, \quad (7)$$

where

$$\begin{aligned} C(\lambda_i, L_{Tur}) &= \frac{-(1 - \beta_i)e^{-\kappa_i L_{Tur}}}{(1 + \beta_i)^2 e^{\kappa_i L_{Tur}} - (1 - \beta_i)^2 e^{-\kappa_i L_{Tur}}} \\ &= -\frac{(1 - \beta_i)e^{-\kappa_i L_{Tur}}}{D_i(L_{Tur})}, \\ B(\lambda_i, L) &= \frac{(1 + \beta_i)e^{\kappa_i L_{Tur}}}{D_i(L_{Tur})}, \\ \beta_i &= \left[\frac{A_{KM}(\lambda_i)}{A_{KM}(\lambda_i) + 2S_{KM}(\lambda_i)} \right]^{1/2}, \\ \kappa_i &= \{A_{KM}(\lambda_i)[A_{KM}(\lambda_i) + 2S_{KM}(\lambda_i)]\}^{1/2}. \end{aligned}$$

Figure 2 illustrates the method that we used to calculate H_{out} , which is a modification of that developed by Gijbbers *et al.*¹⁰ Fluorescence is assumed to be emitted equally in both $+z$ and $-z$ directions. The fraction of fluorescence generated at depth z that reaches the sample front surface is

$$\begin{aligned} H_{out}[\text{op.prop.}(\lambda_j), z, L_{Tur}] &= 1/2[T_z + R_z R_{L_{Tur}-z} T_z + (R_z R_{L_{Tur}-z})^2 T_z + \dots] \\ &\quad + 1/2[R_{L_{Tur}-z} T_z + R_{L_{Tur}-z} R_z R_{L_{Tur}-z} T_z \\ &\quad + R_{L_{Tur}-z} (R_z R_{L_{Tur}-z})^2 T_z + \dots] \\ &= \frac{1}{2} \frac{T_z}{(1 - R_{L_{Tur}-z} R_z)} + \frac{1}{2} \frac{T_z R_{L_{Tur}-z}}{(1 - R_{L_{Tur}-z} R_z)}, \end{aligned} \quad (8)$$

where R and T indicate the reflection and transmission coefficients at the emission wavelength for the tissue layer of thickness given by the subscript.

Fig. 2. Method of calculating H_{out} . Fluorescence is assumed to be emitted equally in the $+z$ direction (dashed arrows) and $-z$ direction (solid arrows). The series approximation of Ref. 10 is used to describe the fraction of fluorescence generated at z that reaches the front surface. $R_z = R_z(\lambda_j)$ and $T_z = T_z(\lambda_j)$ indicate the reflection and transmission coefficients, respectively, at the emission wavelength of the layer of tissue of thickness z above the fluorescing layer. $R_{L_{Tur}-z}$ indicates the reflection coefficient at the emission wavelength of the layer of tissue of thickness $L_{Tur} - z$ below the fluorescing layer.

These can be calculated from Kubelka–Munk theory as^{17,18}

$$R_z(\lambda_j) = \frac{(1 - \beta_j^2)(e^{\kappa_j z} - e^{-\kappa_j z})}{D_j(z)}, \quad (9)$$

$$T_z(\lambda_j) = \frac{4\beta_j}{D_j(z)}.$$

Equation (9) for $R_{L_{\text{Tur}}}$ and $T_{L_{\text{Tur}}}$ can be inverted to give β and κ , and thus A_{KM} and S_{KM} . Combining Eqs. (8) and (9), H_{out} becomes

$$H_{\text{out}} = \frac{[(1 + \beta_j)e^{\kappa_j(L_{\text{Tur}}-z)} - (1 - \beta_j)e^{-\kappa_j(L_{\text{Tur}}-z)}]}{D_j(L_{\text{Tur}})}. \quad (10)$$

The Kubelka–Munk transfer function can then be calculated, yielding

$$\begin{aligned} \text{TF}_{\text{KM}}(\lambda_i, \lambda_j, L_{\text{Tur}}) = & \frac{2}{D_i(L_{\text{Tur}})D_j(L_{\text{Tur}})} \left[\frac{-p_1}{q_1}(e^{-q_1 L_{\text{Tur}}} - 1) \right. \\ & + \frac{p_2}{q_1}(e^{q_1 L_{\text{Tur}}} - 1) + \frac{-p_3}{q_2}(e^{-q_2 L_{\text{Tur}}} - 1) \\ & \left. + \frac{p_4}{q_2}(e^{q_2 L_{\text{Tur}}} - 1) \right], \quad (11) \end{aligned}$$

where

$$p_1 = (\beta_i + 1)(\beta_j + 1)e^{(\kappa_i + \kappa_j)L_{\text{Tur}}},$$

$$p_2 = (\beta_i - 1)(\beta_j - 1)e^{-(\kappa_i + \kappa_j)L_{\text{Tur}}},$$

$$p_3 = (\beta_i + 1)(\beta_j - 1)e^{(\kappa_i - \kappa_j)L_{\text{Tur}}},$$

$$p_4 = (\beta_i - 1)(\beta_j + 1)e^{-(\kappa_i - \kappa_j)L_{\text{Tur}}},$$

$$q_1 = \kappa_i + \kappa_j,$$

$$q_2 = \kappa_i - \kappa_j.$$

In the limit as L_{Tur} approaches infinity (semi-infinite medium), the expression for the TF becomes

$$\text{TF}_{\text{KM}}(\lambda_i, \lambda_j) = \frac{2}{(\beta_i + 1)(\beta_j + 1)(\kappa_i + \kappa_j)}. \quad (12)$$

For an absorption-dominant case $A_{\text{KM}} \gg S_{\text{KM}}$, Eq. (12) reduces to

$$\text{TF}_{\text{KM}}(\lambda_i, \lambda_j) = \frac{1}{2[A_{\text{KM}}(\lambda_i) + A_{\text{KM}}(\lambda_j)]}. \quad (13)$$

The functional form of Eq. (13) is the same as that derived assuming exponential attenuation of light (the Beer law).³

Experimental Validation

A series of experiments was undertaken to investigate how accurately the transfer function of Eq. (11) could deconvolve absorption and scattering effects from the fluorescence of a turbid tissue. An EEM was recorded from a full-thickness tissue specimen as an experimental measure of EEM_{Tur} . Total diffuse fluorescence and reflectance were measured, and Kubelka–Munk optical properties were calculated. The transfer function was calculated and the equivalent-dilute EEM was calculated from Eq. (5). A fluorescence EEM was obtained from a 4- μm -thick sample of the same tissue as an experimental measure of EEM_{Dil} .

The media of human aorta was obtained at autopsy and was chosen for these studies because it contains a homogeneous distribution of fluorophores and has well-characterized optical properties over the UV and visible regions of the spectrum.^{3,16} The media was blunt dissected from the aorta. To increase the absorption coefficient, the media was soaked in diluted, hemolysed blood for approximately 10 min; tissue thickness was measured with a micrometer. Following spectroscopic measurements from the thick

Fig. 3. Emission spectra of 4- μm -thick (dilute) and 250- μm -thick (turbid) sections of media at the 340-nm excitation. Fluorescence intensity has been normalized as to excitation intensity and sample thickness. (The same set of arbitrary units is maintained throughout the paper.)

sample, a cryostat microtome was used to obtain a 4- μm -thick specimen. Fluorescence EEM's for both 250- μm -thick (turbid) and 4- μm -thick (dilute) sections were acquired for an excitation-wavelength range of 300–500 nm in 10-nm increments and for an emission-wavelength range of 310–700 nm in 5-nm increments with a standard scanning spectrofluorimeter (SPEX Fluorolog II, Edison, N.J.). EEM's were

corrected for the nonuniform spectral response of the detection system and were normalized to the excitation intensity, which was measured with a Rhodamine B quantum counter,¹⁹ and to sample thickness. To generate optical properties, diffuse reflectance and transmission were measured from 300–700 nm at 0.5-nm intervals for the 250- μm -thick sample with an absorption spectrophotometer that had an integrating-

Fig. 4. Measurements from a 250- μm -thick section of aortic media: (a) Diffuse reflectance and transmission spectra over the range 300–700 nm, (b) the corresponding Kubelka-Munk absorption and scattering coefficients, (c) the corresponding transport optical properties μ_a and μ_s' where $g = 0.88$.

sphere accessory (Hitachi, Tokyo, Japan). For all spectroscopic measurements, tissue samples were mounted on a piece of Plexiglas over a 1-cm-diameter hole ensuring an air-tissue interface at the front and back surfaces.

Figure 3 illustrates the effect that scattering and reabsorption produce on the emitted fluorescence of turbid tissue. Shown are fluorescence-emission spectra at 340-nm excitation (normalized to excitation

intensity and sample thickness) corresponding to the 4- μm -thick (dilute) and 250- μm -thick (turbid) samples of media. These emission spectra represent a row in the EEM with $\lambda_i = 340 \text{ nm}$. Equation (1) predicts that the normalized fluorescence spectra would be the same for homogeneous dilute samples of varying thickness; however significant differences in absolute intensity and line shape are observed. The peak fluorescence intensity of the turbid sample is approxi-

Fig. 5 continued.

mately three time lower than that of the corresponding dilute sample. In addition, the line shapes are significantly different; the spectrum of the dilute sample shows a single emission peak at 400 nm, while the spectrum of the turbid sample shows two peaks at 390 and 440 nm, with an intervening valley at 410 nm. This is due to the Soret-band absorption of oxyhemoglobin as shown below and in the literature.^{3,10,16}

Figure 4(a) shows diffuse reflection and transmission spectra obtained for the 250- μm -thick sample. Kubelka-Munk absorption and scattering coefficients were calculated for these data and are depicted in Fig. 4(b). For purposes of comparison, the transport optical properties μ_a and μ_s' [Fig. 4(c)], were also calculated assuming an anisotropy factor of 0.88.¹⁰ We made these calculations using inverse-diffusion theory with the delta-Eddington phase function and mismatched boundary conditions.²⁰ The effects of oxyhemoglobin absorption can be seen in μ_a at 410, 540, and 577 nm. These absorption peaks are also present in A_{KM} . Note that the Kubelka-Munk scattering coefficient shows a valley at 410 nm. This indicates the inability of Kubelka-Munk theory to completely separate the effects of absorption and scattering when both the absorption and the scattering coefficients are high.²¹ These effects are not in evidence in the scattering coefficient calculated from inverse-diffusion theory, indicating that the diffusion theory is better able to account for the physical effects typically seen in turbid tissues.

The Kubelka-Munk optical properties shown in Fig. 4(b) were used to calculate the transfer function, according to Eq. (11). The Kubelka-Munk transfer function is illustrated in Fig. 5(a) for the 340-nm excitation. The effects of reabsorption at 410, 540, and 577 nm are observed as minima in the transfer function at these particular emission wavelengths. The equivalent dilute-emission spectrum at 340-nm

excitation was calculated from Eq. (6) with the use of this transfer function and the measured turbid-emission spectrum shown in Fig. 3. The results are illustrated in Fig. 5. Figure 5(b) shows the measured turbid-emission spectrum and the measured turbid- and dilute-emission spectra, all normalized to sample thickness and excitation intensity. Agreement between the intensities of the measured and calculated dilute-fluorescence spectra is good, within 5% at wavelengths below 410 nm, and within 20% at all wavelengths. Figure 5(c) provides a clearer comparison of the differences in the fluorescence line shape of the measured turbid-emission and measured and calculated dilute-emission spectra; these data are shown normalized to 1 at 460 nm. Although the transfer function was significantly corrected for oxyhemoglobin reabsorption at this wavelength, the predicted dilute-emission spectrum still shows some residual reabsorption at 415 nm. Figure 5(d) shows measured turbid-emission and measured and calculated dilute-emission spectra at an additional excitation wavelength of 450 nm. Here, the contributions of reabsorption to the turbid-emission spectrum are not as great, with shallow valleys at 540 and 577 nm. The calculated and measured dilute-emission spectra agree within 10% at all wavelengths. These data are representative of the agreement observed over the entire excitation-wavelength range spanned by the EEM.

Simulations

To assess the sensitivity of the transfer function to its component optical properties, the transfer function was calculated as a function of A_{KM} and S_{KM} for a range of optical properties typical for tissues. To explore the dependence of the semi-infinite transfer function [Eq. (12)] on S_{KM} , A_{KM} was held constant at a value that is typical for a sample of aorta at 340-nm excitation and 590-nm emission, while S_{KM} was var-

Fig. 5. Comparison of measured and calculated data: (a) the transfer function for the 340-nm excitation; (b) measured turbid- and dilute-emission spectra at the 340-nm excitation wavelength compared with the calculated dilute-emission spectrum based on the transfer function (a); (c) measured turbid- and dilute-emission spectra at the 340-nm excitation compared with the calculated dilute-emission spectrum where all data has been normalized to a value of 1 at 460 nm, indicated by the arrow; (d) measured turbid- and dilute-emission spectra at the 450-nm excitation compared with the calculated dilute-emission spectrum.

ied from 100 times less to 10 times more than the value of S_{KM} for aorta at both the same excitation and emission wavelengths. Similarly, to assess the sensitivity of the transfer function to variations in A_{KM} , S_{KM} was held constant and A_{KM} was varied from 100 times less to 10 times more than the value of A_{KM} for aorta, at the same excitation and emission wavelengths. The results are shown in Fig. 6. These plots indicate that the transfer function is much more sensitive to changes in the absorption coefficient than to changes in the scattering coefficient. This implies that errors related to the measurement of absorption properties must be minimized to determine the transfer function accurately. Conversely, the transfer function is relatively immune to inaccuracies in the determination of the scattering coefficient.

Conclusions and Future Directions

This paper presents a general relationship between the fluorescence of dilute and turbid samples. This relationship, known as the transfer function, is independent of the model of propagation used to describe the behavior of light within the sample. The trans-

fer function can remove the effects of scattering and reabsorption from the fluorescence of turbid samples. Data from turbid samples can then be analyzed with methods such as RAFA that have been developed for dilute solutions.

The form of the transfer function was derived using the two-flux Kubelka-Munk theory. It was demonstrated that the transfer function can be calculated from measurements of diffuse transmission and reflection. The ability of the Kubelka-Munk transfer function to deconvolve scattering and absorption effects was illustrated with measured data from samples of human aorta. At the 450-nm excitation wavelength, agreement between the intensities of the measured and calculated dilute-fluorescence spectra was excellent, within 10% at all wavelengths. Agreement between the intensities of the measured and predicted dilute-fluorescence spectra was also good, within 5% at wavelengths below 410 nm, and within 20% at all wavelengths. Although the transfer function significantly corrects for oxyhemoglobin reabsorption at this wavelength, the predicted dilute-emission spectrum still shows some residual reabsorption at

Fig. 6. Sensitivity of the transfer function to the scattering and absorption coefficients: (a) the transfer function as a function of S_{KM} while A_{KM} is held constant (see text); the arrow indicates the value of S_{KM} for aorta at 340 nm; (b) the transfer function as a function of A_{KM} while S_{KM} is held constant; the arrow indicates the value of A_{KM} for aorta at 340 nm. Note the difference of a factor of 10 in the y axes.

410 nm. This agreement between measured and calculated dilute-emission spectra over a wide range of excitation wavelengths is particularly noteworthy because the model requires no assumptions about the tissues optical properties and has no fittable parameters. Although the transfer function presented here can be calculated from diffuse reflectance and transmission, it has an explicit optical-property dependence. Simulations were performed that indicate that the transfer function is much more sensitive to the absorption coefficient than to the scattering coefficient.

An analytic model based on photon migration has recently been described, and it permits calculation of a dilute solution's fluorescence-emission line shape from measurements of a turbid sample's fluorescence-emission line shape and diffuse-reflectance spectrum of a homogeneous, semi-infinite sample.²² This model was tested with 476-nm excited-fluorescence spectra of human aorta. Although the photon-migration model completely removed effects of reabsorption when values of the tissue scattering coefficient and anisotropy were assumed, it provides only line-shape and not intensity information. Thus, analytic methods developed for dilute solutions can be used in conjunction with the photon-migration model, but they will yield only relative tissue-chromophore contributions and not absolute concentrations.

The model presented here provides absolute intensity information with no assumptions about optical properties and thus gives a method that extends RAFA techniques to the analysis of fluorescence EEM's from turbid materials. Future work will explore the accuracy of this modified RAFA technique based on Kubelka-Munk theory. Finally, it should be noted that the concept of the transfer function is general, and can be used with more sophisticated models of light propagation. Of particular interest is the application of the three-dimensional diffusion approximation to a case in which the sample under investigation is optically thick and has optical properties that can be determined from measurements of radial reflectance.²³

Appendix A: Review of RAFA

Techniques for applying rank-annihilation-factor analysis (RAFA) to fluorescence EEM's have been described extensively in the literature; the procedure will only be outlined here.¹⁵ The RAFA technique is based on a description of a fluorescence EEM of a dilute solution as follows: The fluorescence EEM of a dilute solution with N fluorophores is a matrix that contains the detected fraction of the total fluorescence intensity $S_{Total}(\lambda_i, \lambda_j)(\Omega/4\pi)$ normalized to the excitation intensity $I(\lambda_i)$ and the sample thickness L_{dil} . The fraction of fluorescence detected is given by Ω , the solid angle of collection of the detector, over the 4π total solid angle, assuming an isotropic emission of fluorescence. The ij th element of this matrix is

given by

$$\begin{aligned} EEM_{Dil_{ij}} &= \left(\frac{\Omega}{4\pi} \right) \frac{S_{Total}(\lambda_i, \lambda_j)}{I(\lambda_i)L_{dil}} \\ &= \left(\frac{\Omega}{4\pi} \right) \sum_{k=1}^N 2.3C_k \epsilon_k(\lambda_i) \phi_k(\lambda_j) \\ &= \left(\frac{\Omega}{4\pi} \right) \sum_{k=1}^N \mu_{a_k}(\lambda_i) \phi_k(\lambda_j), \end{aligned} \quad (A1)$$

where each fluorophore can be characterized by a concentration C_k , a wavelength-dependent molar-absorption coefficient, $\epsilon_k(\lambda_i)$, and a wavelength-dependent fluorescence quantum yield, $\phi_k(\lambda_j)$.⁹

Equation (A1) can be rewritten in matrix notation as

$$EEM_{Dil} = X\Phi^T, \quad (A2)$$

where EEM_{Dil} is an $m \times p$ matrix containing fluorescence intensities at the m -excitation and p -emission wavelengths, X is an $m \times N$ matrix, the columns of which are given by $2.3C_k \epsilon_k$ at the m -excitation wavelengths, and Φ is a $p \times N$ matrix, the columns of which contain $(\Omega/4\pi)\phi_k$ at the p -emission wavelengths. The ranks of X and Φ are given by the number of independent emitters N . Therefore, the rank of EEM_{Dil} is also N .¹²

The RAFA technique is based on the observation that when the fluorescence contribution of a pure fluorophore is exactly subtracted from an EEM of a solution containing that fluorophore and other unknowns, the rank of the resulting matrix will drop by one. If the EEM measured for a dilute mixture containing an unknown concentration of chromophore A, EEM_{Mix} , and the EEM of a solution containing a known concentration of only chromophore A, EEM_A are measured, then the difference

$$EEM_{Mix}' = EEM_{Mix} - C_A(EEM_A), \quad (A3)$$

will have rank $N - 1$ when the constant C_A is equal to the ratio of the concentration of component A in the unknown mixture and in the pure solution.

Both iterative and analytic techniques have been proposed for calculating C_A .¹¹⁻¹⁵ Here, we describe an analytic method first presented by Lorber.¹⁴ Mathematically, the reduction of the rank of a matrix can be formulated as

$$\det|EEM_{Mix}'| = \det|EEM_{Mix} - C_A EEM_A| = 0 \quad (A4)$$

Lorber has shown that Eq. (A4) can be rewritten in the exact form of the eigenvalue-eigenvector problem yielding an analytic expression for C_A :

$$C_A = \frac{1}{\text{Tr}(ab^T S^{-1})} \quad (A5)$$

where $a = U^T x_A$, $b = V^T \phi_A$, when EEM_{Mix} is written in terms of its singular value decomposition, $EEM_{Mix} = U S V^T$.

Thus, the technique of RAFA provides a method of quantifying the concentration of a fluorescent compound with known optical properties, (x_A, ϕ_A) from the EEM of a dilute solution containing an unknown number of chromophores with unknown optical properties (EEM_{Mix}). This method is based on Eq. (A1), which assumes that the fluorescence of a sample can be described as a dilute solution.

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