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ORIGINAL PAPER

Nonlinear Behavior of the Autofluorescence Intensity on the Surface of Light-Scattering Biotissues and its Theoretical Proof

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Abstract In the up-to-date medical laser fluorescence spectroscopy (LFS) in vivo, there is a problem of quantification of fluorophores concentrations in optically-turbid biotissues by measurements of the laser induced autofluorescence flux on the surface of these tissues. One of the main problems is: whether the flux depends linearly or non-linearly on the concentration of fluorophores in tissues? The purpose of this work was both experimental and theoretical study of the character of dependencies between measured fluorescence intensities and fluorophores concentrations in optically-turbid media. In the experimental part of our study, measurements of the superficial fluorescence on phantoms at various known concentrations of fluorophores in them were carried out. As a result, experimental dependencies of registered intensities of the laser-induced autofluorescence emission were plotted against fluorophore concentrations. In the theoretical part of the study, the analytical solution for the fluorescence emission by Kokhanovsky's method based on the well-known two-flux Kubelka-Munk approach (KMA) was used. In addition, in our study the Kokhanovsky's method was modified by its association with our improved KMA, allowing us to receive exact analytical solutions for boundary intensities collected by optical probes. As a result, a set of theoretical curves describing the influence of fluorophore concentrations on the registered autofluorescence intensities was obtained, as well. Both experimental and theoretical results show a good qualitative agreement with each other. Also, these results demonstrate that the dependence of the fluorescence intensity on tissues' optical properties and on the concentration of fluorophores in light-scattering tissues can be both nonlinear and non-monotonic.

Keywords Autofluorescence · Flux · Fluorophore · Concentration · Noninvasive · Diagnostics · Theoretical simulations

Introduction

Laser fluorescence spectroscopy (LFS) has been presented as a sensitive technique for in vivo characterization of tissues for diagnosis purposes in medicine [1–4]. One of the objectives of LFS is to evaluate the fluorophores' concentration (both exogenous and endogenous) in the inspected volume of tissues [3]. For example, the efficacy of any photodynamic therapy (PDT) is highly dependent on a photosensitizer concentration present in the tumor [5]. Another aspect is associated with assessment of cutaneous collagen and elastin content in tissues in normal and pathologic conditions [6]. Collagen and elastin are endogenous fluorophores which have a specific fluorescence and account for up to 70 % of total cutaneous proteins. Therefore, they are very convenient optical markers for structural cutaneous anomalies [7]. In such skin disorders as scleroderma, basal-cell carcinoma, keloid scars, etc., the balance between collagen synthesis and degradation is impaired, so in vivo quantitative assessment of the dermal collagen can be very promising. Also, in the past few years another interesting problem has been brought to attention. It is in vivo investigation of pharmacokinetics of various drug forms with fluorescent labels in human and animal organism [4]. On the one hand, it is associated with studies of new drugs produced as nano- or microcapsules which are considered to be promising targeted treatment and could be potentially used for

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synthesis of drugs for prolonged administration. On the other hand, when the clinical tests of drugs are carried out, there is always a question as to whether a patient really takes the drug as prescribed by doctors or secretly avoids it, doesn't follow the regimen or forgets to take them on time. Therefore, a non-invasive modality to monitor patient's compliance with medication is quite urgent necessity. In all these applications, a quantification of any fluorophores concentration is extremely important. Without this option all commercial LFS systems are qualitative only and, therefore, are purely subjective. But in the up-to-date medical LFS in vivo, there is a problem of quantification of fluorophores content in optically-turbid biotissues if measurements of the laser induced fluorescence (autofluorescence) are carried out on the tissue surface [3]. These measurements don't allow a physician to directly register the fluorophore concentration. LFS implies several indirect optical techniques and approaches, so special computation algorithms are needed to extract the information about the concentration from the registered optical signal [5].

One of the main problems on this way is a complex nature of dependencies of registered fluorescence intensities on optical properties of tissues at light-scattering. A number of authors have repeatedly attempted to construct different theoretical models to calculate the concentration and to develop algorithms for correction of measured fluorescent spectra attenuated and distorted by other substances in tissues, such as haemoglobin, melanin, etc. [5, 8–10] In particular, it has been shown that the fluorescence signal recorded in vivo from biological tissues is strongly affected by scattering and absorption inside the tissue and, thus, is not directly related to the fluorophore concentration [11]. However, in our opinion, the question of the linearity or nonlinearity of dependencies of the registered fluorescence signal on the fluorophore concentration in tissues is not fully elucidated and is not clear enough in all these publications. Moreover, until recently, all such works were based on some approximate methods of solving the transport problem - method of moments [9], diffusion approximation [10], simplified heuristic algorithms [5, 12] or on the basis of the well-known statistical algorithm of a random walk of a photon in the medium (Monte Carlo simulation method [13]). For example, in Ref. [5], a fluorophore concentration was quantified by a semi-heuristic Gardner's model [14] that corrects a raw fluorescence signal by compensating for optical absorption (μ_a) and scattering (μ_s) losses both at excitation and emission wavelengths. Each of these approaches has certain drawbacks. Heuristic algorithms, as a rule, are applicable to a chosen design of the diagnostic device only. Approximate solutions possess low accuracy, while numerical methods like the Monte Carlo simulation require extensive calculations and do not provide a solution in the form of a closed analytical expression which could be easily analyzed for how one or another optical parameter affects the final registered fluorescent spectrum.

Recently Kokhanovsky proposed a rigorous analytical method based on the classical two-flux Kubelka–Munk (KM) approximation to solve the fluorescence problem [15]. Also, the author of Ref. [16] showed the main source of errors of the classic KM model and proposed a generally improved version of KM equations which made it possible in one-dimensional (1D) problems to obtain more accurate values for radiation fluxes at boundaries of the light-scattering medium (for backscattered and transmitted fluxes detected by an optical probe of the diagnostic equipment). This opens up some prospects of constructing simple analytical and real-time algorithms to analyze the distortions of fluorescence spectra without resorting to complex multi-step computations and numerical methods [17]. But in the articles above mentioned, the problem of linearity or nonlinearity of the registered fluorescence signal dependence on the fluorophore concentration in optically-turbid tissues was not considered as well. So, the aim of the present our study is both experimental and theoretical evaluation of the character of dependencies of measured fluorescence intensities on fluorophore concentrations in optically-turbid media.

Experimental Setup and Results

Aluminum phthalocyanine-based photosensitizer "Photosens", as well as porphyrin-based photosensitizer "Radahlorin" were used as fluorophores. Two different fluorophores were selected for testing in order to obtain general dependencies, which are non specific to nature of each of them. Solutions with various concentrations C_f of these fluorophores were placed in self-made light-scattering phantoms (measures). Several constructive and functional features of the measures are presented in Fig. 1. Photosensitizer solutions 1 are filled in poles (cavities) in the solid light scattering foundation 2. The poles are closed by a light scattering plate 3,

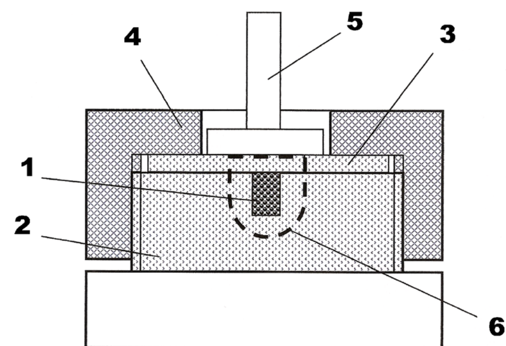

Fig. 1 Constructive and functional features of measures: **1** – cavity with photosensitizer solutions, **2** – light-scattering foundation, **3** – light-scattering plate, **4** – lid with a window for an optical fiber probe, **5** – the optical fiber probe steady tip, **6** – sampling (diagnostic) volume at measurements

on which an optical fiber probe steady tip 5 is located. The frontal lid 4 with a window for the optical fiber probe closes and firmly holds all construction. Ideologically, such a design can simulate a fluorescence of a tumor inside light-scattering biological tissues.

To induce and register fluorescence of photosensitizers, a commercial laser-based multifunctional noninvasive medical diagnostic system "LAKK-M" [18] was used (Fig. 2, on the left). The fluorescence excitation was made by the "red" semiconductor laser with the wavelength λ=635 nm and light power of 5 mW. Laser radiation was delivered to the measure by a multimode optical fiber. Fluorescence flux was registered in the waveband 650–680 nm by the built-in fiber optic spectrometer with the CCD detector, which was included in the system. To avoid trembling of the optical fiber probe, it was mounted in a stand and fixed during all measurements (Fig. 2, on the right).

As a result, experimental relationships between the registered superficial fluorescence intensity and the concentration of fluorophores in light-scattering measures were plotted (Fig. 3).

It can be seen that all autofluorescent fluxes on the surface of the tested light-scattering medium nonlinearly depend on the fluorophore concentration in the medium. Moreover, this nonlinearity is also evidently manifested for very low concentrations, therefore, there must exist weighty fundamental reasons for that.

Theoretical Approach and Results

It is obvious that the registered nonlinear phenomenon must have a theoretical proof and explanation, as well. To obtain one, we used the exact analytical solution for fluxes of fluorescence emission on the surface of the light-scattering medium by Kokhanovsky's method [15], which is based, in its turn, on the well-known two-flux Kubelka-Munk (KM) approach. Also, the Kokhanovsky's method was developed and expanded by means of our ideas of the generally improved KM approximation [17]. While initially the classic KM equations are a two-flux 1D theory, there are only two directions in the medium for light propagation – forward and backward (upward and downward in the experimental setup). Following the Kokhanovsky's designations, let the forward-directed flux at the excitation wavelength λ_e be denoted by i(x), and the backward-directed one be denoted by j(x). Let the corresponding fluorescent fluxes at the wavelength of fluorescence, λ_f, be denoted as I(x) and J(x) respectively. Also, let the coordinate axis "x" be directed forward (from left to right, see Fig. 4).

In the task we assume that the incident (excitation) monochromatic light with the excitation flux Φ_0 at the wavelength λ_e illuminates the left surface (x=0) of a tested light-scattering medium. Inside the medium some part of the excitation flux induces fluorescence at the wavelength λ_f, so that both the pure backscattered radiation j(0) at λ_e and the fluorescent backscattered radiation J(0) at λ_f can be detected from the left surface of the medium. Also, we assume that the medium is semi-infinite, so there is no light incident on the right side of it. Unlike the Kokhanovsky's model, in our approach there is no light incident on the left boundary of the medium at any fluorescence wavelengths λ_f as well.

From the optical point of view, within the limits of the used improved KM approach [16], the examined 1D light-scattering medium is characterized by its local optical properties: absorption coefficient $\mu_a(\lambda)$ [mm^{-1}], reflection (Fresnel's) coefficient R(λ), characterizing the reflection on the boundaries of inhomogeneities, and the average density of these inhomogeneities μ_ρ [mm^{-1}] inside the medium. Using the Kokhanovsky's approach ideology [15] together with the generally improved KM approach [16], all these statements can be written mathematically as the basic system of differential equations describing the forward I(x) and backward J(x) fluorescent fluxes as follows [17]:

$$\begin{cases} dI(x)\big/dx = -\beta_1(\lambda_f)\cdot I(x) + \beta_2(\lambda_f)\cdot J(x) + F_{ef}(x) \\ dJ(x)\big/dx = \beta_1(\lambda_f)\cdot J(x) - \beta_2(\lambda_f)\cdot I(x) - F_{ef}(x) \end{cases}, \quad (1)$$

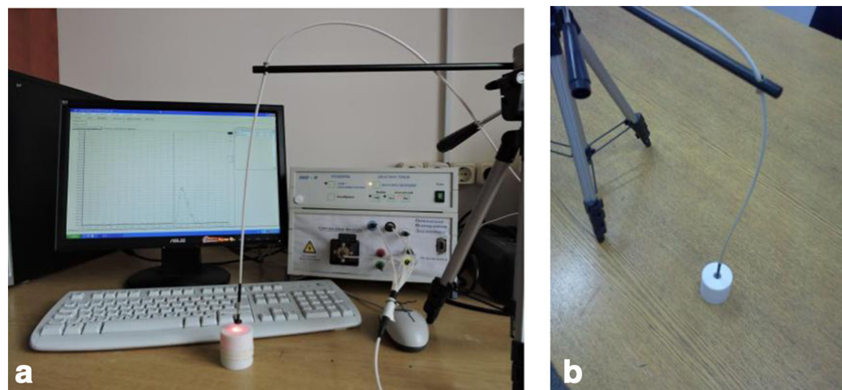

Fig. 2 Multifunctional noninvasive laser diagnostic system "LAKK-M" with a multiple fiber optical probe (**a**); optical fiber probe is mounted in a stand and fixed during measurements (**b**)

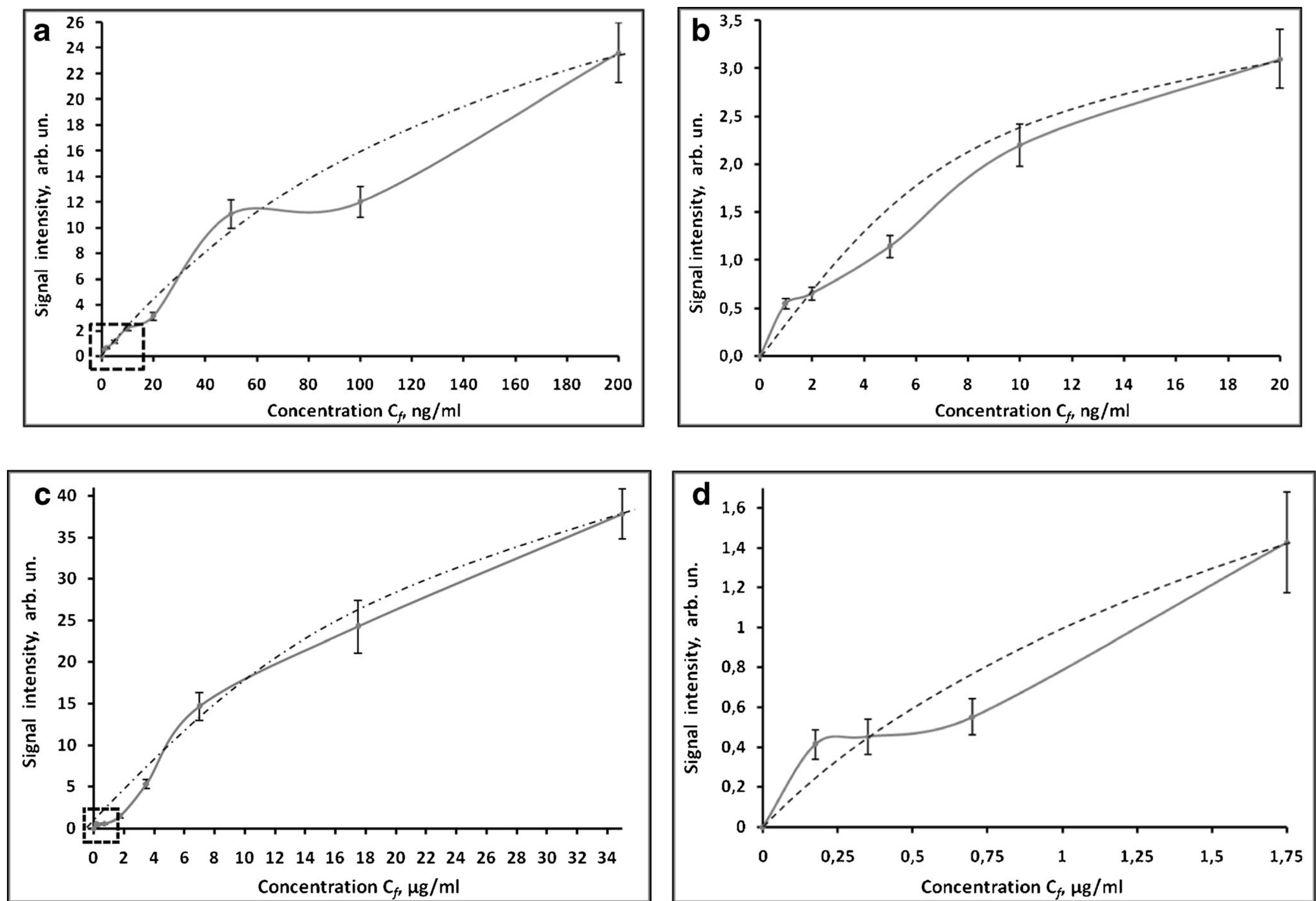

Fig. 3 Measured fluorescence signal intensities versus the concentration of photosensitizers in measures (*solid curves*) and fitting curves (*dashed curves*): (**a**), (**b**) – photosensitizer "Photosens"; (**c**), (**d**) - photosensitizer "Radahlorin"; on the *left* ((**a**), (**c**)) – the baseline dependencies for each photosensitizer; on the *right* ((**b**), (**d**)) - the same signal intensities for the same photosensitizers at low concentrations located in a *dotted square* on the *left*

where $\beta_1(\lambda_f)$ [mm^{-1}] and $\beta_2(\lambda_f)$ [mm^{-1}] are local attenuation and backscattering coefficients of the tested medium at the wavelength λ_f respectively [6]. They are determined for any wavelength λ by the following equations [16]:

$$\beta_1(\lambda) = \omega \cdot \frac{\mu_a(\lambda) - \mu_\rho \ln(1-R(\lambda)) + \mu_\rho \ln\left(1-\omega(\lambda) + \sqrt{\omega^2(\lambda) - R^2(\lambda)e^{-2\mu_a(\lambda)/\mu_\rho}}\right)}{\sqrt{\omega^2(\lambda) - R^2(\lambda)e^{-2\mu_a(\lambda)/\mu_\rho}}},$$
$$\beta_2(\lambda) = R(\lambda)\cdot e^{-\mu_a(\lambda)/\mu_\rho} \cdot \frac{\mu_a(\lambda) - \mu_\rho \ln(1-R(\lambda)) + \mu_\rho \ln\left(1-\omega(\lambda) + \sqrt{\omega^2(\lambda) - R^2(\lambda)e^{-2\mu_a(\lambda)/\mu_\rho}}\right)}{\sqrt{\omega^2(\lambda) - R^2(\lambda)e^{-2\mu_a(\lambda)/\mu_\rho}}} \tag{2}$$

where $\omega(\lambda) = \frac{1-(1-2R(\lambda))\cdot e^{-2\mu_a(\lambda)/\mu_\rho}}{2}$.

Function $F_{ef}(x)$ in Eq. (1) describes the effect of enhanced fluxes due to the fluorescence effect at the wavelength λ_f under the fluorescence excitation at the wavelength λ_e. It can be calculated as [17]:

$$F_{ef}(x) = \frac{1}{2} A_f(\lambda_e) \cdot \varphi(\lambda_e, \lambda_f) \cdot \Phi_0 \cdot (1 + r_{\infty\lambda e}) \cdot e^{-\alpha_{\lambda e} x}, \tag{3}$$

where $\varphi(\lambda_e, \lambda_f)$ is a quantum efficiency of the fluorescence yield; $\alpha_{\lambda e} = \sqrt{\beta_1^2(\lambda_e) - \beta_2^2(\lambda_e)}$ and $r_{\infty\lambda e} = \frac{\beta_2(\lambda_e)}{\beta_1(\lambda_e) + \alpha_{\lambda e}}$.

Coefficient $A_f(\lambda_e)$ [mm^{-1}] in Eq. (3) describes a part of total excitation radiation {i(x)+j(x)} which is absorbed by the fluorophore distributed inside the elementary path-length "dx" in the medium. It should be specially noted that in the general case, $A_f(\lambda_e)$ is not equal (see explanations below) to

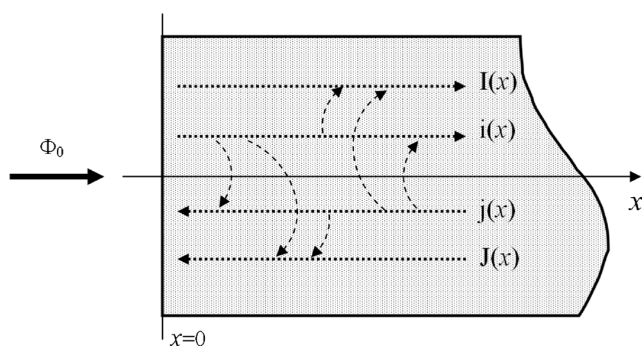

Fig. 4 Formulation of the 1D problem of light propagation in the light-scattering medium with fluorescence (see text for explanation)

the absorption coefficient $\mu_{af}(\lambda_e)$ of the fluorophore, unlike it is usually accepted at other similar calculations, in the original Kokhanovsky's model [15], for example. We have found out this statement in the current study only, so in our previous article [17] there was a mistake in the similar equation.

To introduce into consideration a concentration C_f of the fluorophore in relative units ($0<C_f<1$), the total absorption coefficient $\mu_{a\Sigma}(\lambda_e)$ of the medium at the excitation wavelength λ_e was expressed as the sum:

$$\mu_{a\Sigma}(\lambda_e) = \mu_{af}(\lambda_e) + \mu_{at}(\lambda_e) = \mu_{af}(\lambda_e)_{max} C_f + \mu_{at}(\lambda_e)_{max}(1-C_f), \quad (4)$$

where: $\mu_{af}(\lambda_e)=\mu_{af}(\lambda_e)_{max}C_f$ is the absorption coefficient of the fluorophore at the wavelength λ_e, C_f is the relative concentration of the fluorophore in the medium, $\mu_{af}(\lambda_e)_{max}$ is the maximum absorption coefficient of the fluorophore at the wavelength λ_e in the limit case $C_f=1$, $\mu_{at}(\lambda_e)=\mu_{at}(\lambda_e)_{max}(1-C_f)$ is the absorption coefficient of the light-scattering medium matrix at the wavelength λ_e, $\mu_{at}(\lambda_e)_{max}$ is the maximum absorption coefficient of the light-scattering medium matrix in the case of the pure medium (without any fluorophores) at the wavelength λ_e. The same approach was used to describe the total absorption coefficient, $\mu_{a\Sigma}(\lambda_f)$, of the medium at the fluorescence wavelength λ_f. However, we considered the case of $\mu_{af}(\lambda_f)_{max}<\mu_{at}(\lambda_f)_{max}$ as well as the case of low fluorophore concentrations ($C_f<<1$). So, under these assumptions one can found that

$$\mu_{a\Sigma}(\lambda_f) \approx \mu_{at}(\lambda_f) \approx \mu_{at}(\lambda_f)_{max} = const.$$

The similar approach was used to describe the total average density, $\mu_{\rho\Sigma}$, of scatterers in the medium:

$$\mu_{\rho\Sigma} = \mu_{\rho f} + \mu_{\rho t} = \mu_{\rho f max} C_f + \mu_{\rho t max}(1-C_f) \quad (5)$$

The average density of inhomogeneities ($\mu_{\rho\Sigma}$) inside the medium is not a function of any wavelength λ. So, to simplify the subsequent numerical calculations without loss of generality, we also further assumed $\mu_{\rho f max}=\mu_{\rho t max}=\mu_\rho$, that, in turn, leads to $\mu_{\rho\Sigma}=const=\mu_\rho$.

By substituting of Eqs. (4)–(5) in Eq. (2), it is possible to calculate $\beta_1(\lambda_e)$ and $\beta_2(\lambda_e)$ at the excitation wavelength λ_e. As long as the fluorescence is excited by radiation which is absorbed by fluorophores only, and because in the frame of our approach the absorption is described as subtraction $\beta_1(\lambda_i)$-$\beta_2(\lambda_i)$, one can obtain that $A_f(\lambda_e)$ should be correctly determined as the difference between values of these two coefficients at nonzero concentration of the fluorophore C_f in the medium and at its zero concentration:

$$A_f(\lambda_e) = \{\beta_1(\lambda_e) - \beta_2(\lambda_e)\}_{C_f\neq 0} - \{\beta_1(\lambda_e) - \beta_2(\lambda_e)\}_{C_f=0} \quad (6)$$

As one can see in the Fig. 5, in this case $A_f(\lambda_e)$ is not equal to $\mu_{af}(\lambda_e)$, i.e. theirs ratio

$$W = A_f(\lambda_e) \big/ \mu_{af}(\lambda_e)$$

is not equal to 1. It can be explained by the fact that in the light-scattering medium the absorbed radiation inside the elementary path-length "dx" is enhanced in comparison to $\mu_{af}(\lambda_e)$ due to multiple scattering inside "dx". Moreover, for a number of cases, the difference between $\mu_{af}(\lambda_e)$ and $A_f(\lambda_e)$ can reach 15-16 % (see Fig. 5). It is so due to the ratio W nonlinearly depends on the concentration C_f. Thus, in the general case, the choice of $A_f(\lambda_e)=\mu_{af}(\lambda_e)$ can lead to some mistakes in calculations up to the level of 15–16 %.

In the practical situation of optically semi-infinite tissues at the noninvasive (in vivo) LFS, as it was mentioned above, the registered fluorescence signal is J(0). Resolving the system (1) with the use of Eqs. (2)–(6) for the asymptotical semi-infinite case with boundary conditions I(0)=0, i(0)=Φ_0, J(∞)=0 and j(∞)=0, one can obtain:

$$J(0) = \Phi_0 \cdot A_f(\lambda_e) \cdot \varphi(\lambda_e, \lambda_f) \cdot \frac{(1+r_{\infty\lambda e})(1+r_{\infty\lambda f})}{2(\alpha_{\lambda e} + \alpha_{\lambda f})} \quad (7)$$

As we can see from Eq. (7), there is a complex dependence of the registered fluorescence flux J(0) on the local optical properties of the inspected tissues at both wavelengths λ_e and λ_f. The registered fluorescence spectra as a function of λ_f depend not only as a complex function $A_f(\lambda_e)$ on the existed fluorophore concentration in the medium, but also depend on the parameter γ [mm]

$$\gamma = \frac{(1+r_{\infty\lambda e})(1+r_{\infty\lambda f})}{2(\alpha_{\lambda e} + \alpha_{\lambda f})}, \quad (8)$$

which describes complex optical properties of the medium, including the concentration C_f, as well. Thus, the expected dependence of the fluorescence flux J(0) on C_f cannot be linear in principle. As an example, in Fig. 6 the calculated flux J(0) versus C_f is presented for

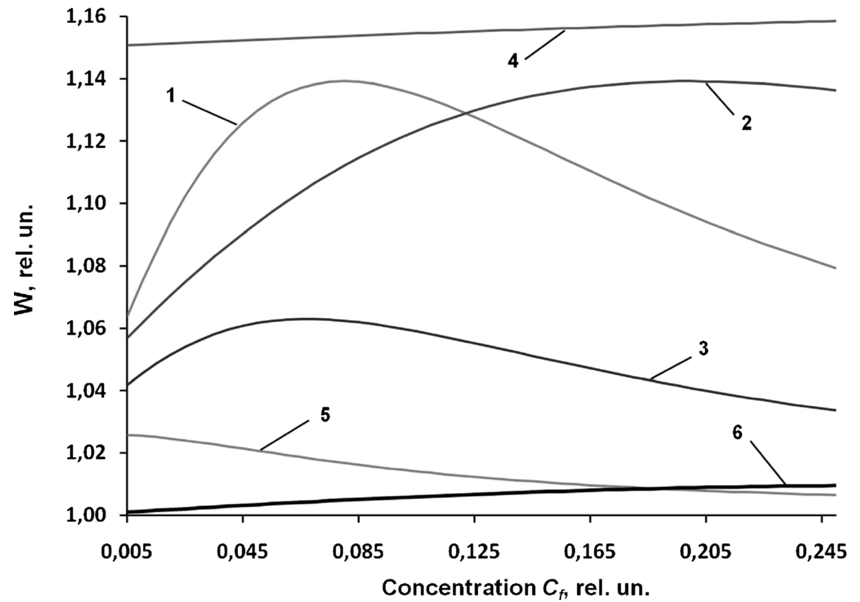

Fig. 5 The ratio W versus the fluorophore concentration C_f: **1**. $\mu_{af}(\lambda_e)_{max}$=50 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=0,5 mm^{-1}; $\mu_{\rho\Sigma}$=2 mm^{-1}; R(λ_e)=0,4. **2**. $\mu_{af}(\lambda_e)_{max}$=20 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=0,5 mm^{-1}; $\mu_{\rho\Sigma}$=2 mm^{-1}; R(λ_e)=0,4. **3**. $\mu_{af}(\lambda_e)_{max}$=100 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=2 mm^{-1}; $\mu_{\rho\Sigma}$=4 mm^{-1}; R(λ_e)=0,2. **4**. $\mu_{af}(\lambda_e)_{max}$=20 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=15 mm^{-1}; $\mu_{\rho\Sigma}$=15 mm^{-1}; R(λ_e)=0,4. **5**. $\mu_{af}(\lambda_e)_{max}$=200 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=10 mm^{-1}; $\mu_{\rho\Sigma}$=6 mm^{-1}; R(λ_e)=0,08. **6**. $\mu_{af}(\lambda_e)_{max}$=80 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=0,5 mm^{-1}; $\mu_{\rho\Sigma}$=10 mm^{-1}; R(λ_e)=0,04

$0<C_f<0,025$. Calculations were made at Φ_0=1 and $\varphi(\lambda_e, \lambda_f)$=1. Even for such rather low relative concentrations (C_f<0,025) all calculated curves are not linear and are similar to our experimental curves in Fig. 3.

By assuming low concentrations of all fluorophores in the medium equal to those in real living biological tissues, analysis of the influence of local optical properties of the medium on parameter γ can be also carried out. For example, Fig. 7 shows how local optical properties nonlinearly influence γ.

Discussion

First of all, we have to note that the experimentally measured J(0) as a function of the fluorophore concentration C_f was registered as nonlinear in all our experiments (see Fig. 3). The similar result was obtained at theoretical calculations of J(0) with the use of our theoretical approach (Fig. 6). Our theoretical result proves and explains this nonlinearity. In our opinion, it is very important because in a lot of current

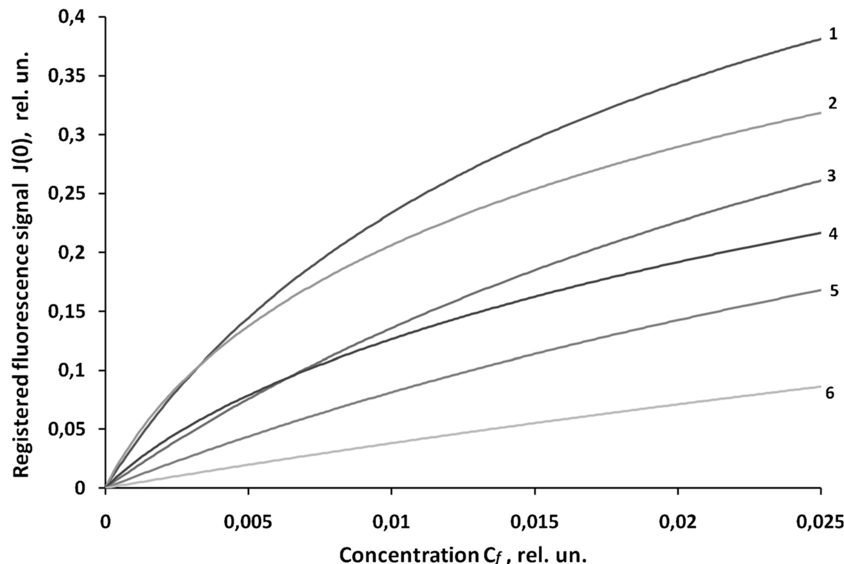

Fig. 6 Registered fluorescence signal J(0) versus relative fluorophore concentration C_f : **1**. $\mu_{af}(\lambda_e)_{max}$=200 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=1 mm^{-1}; $\mu_{\rho\Sigma}$=150 mm^{-1}; R(λ_e)=0,02; $\mu_{a\Sigma}(\lambda_f)$=1 mm^{-1}; R(λ_f)=0,02. **2**. $\mu_{af}(\lambda_e)_{max}$=200 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=0,3 mm^{-1}; $\mu_{\rho\Sigma}$=150 mm^{-1}; R(λ_e)=0,08; $\mu_{a\Sigma}(\lambda_f)$=1 mm^{-1}; R(λ_f)=0,01. **3**. $\mu_{af}(\lambda_e)_{max}$=150 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=3 mm^{-1}; $\mu_{\rho\Sigma}$=150 mm^{-1}; R(λ_e)=0,02; $\mu_{a\Sigma}(\lambda_f)$=1 mm^{-1}; R(λ_f)=0,02. **4**. $\mu_{af}(\lambda_e)_{max}$=200 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=0,3 mm^{-1}; $\mu_{\rho\Sigma}$=500 mm^{-1}; R(λ_e)=0,08; $\mu_{a\Sigma}(\lambda_f)$=2 mm^{-1}; R(λ_f)=0,02. **5**. $\mu_{af}(\lambda_e)_{max}$=150 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=5 mm^{-1}; $\mu_{\rho\Sigma}$=150 mm^{-1}; R(λ_e)=0,08; $\mu_{a\Sigma}(\lambda_f)$=1 mm^{-1}; R(λ_f)=0,01. **6**. $\mu_{af}(\lambda_e)_{max}$=100 mm^{-1}; $\mu_{at}(\lambda_e)_{max}$=5 mm^{-1}; $\mu_{\rho\Sigma}$=500 mm^{-1}; R(λ_e)=0,08; $\mu_{a\Sigma}(\lambda_f)$=2 mm^{-1}; R(λ_f)=0,04

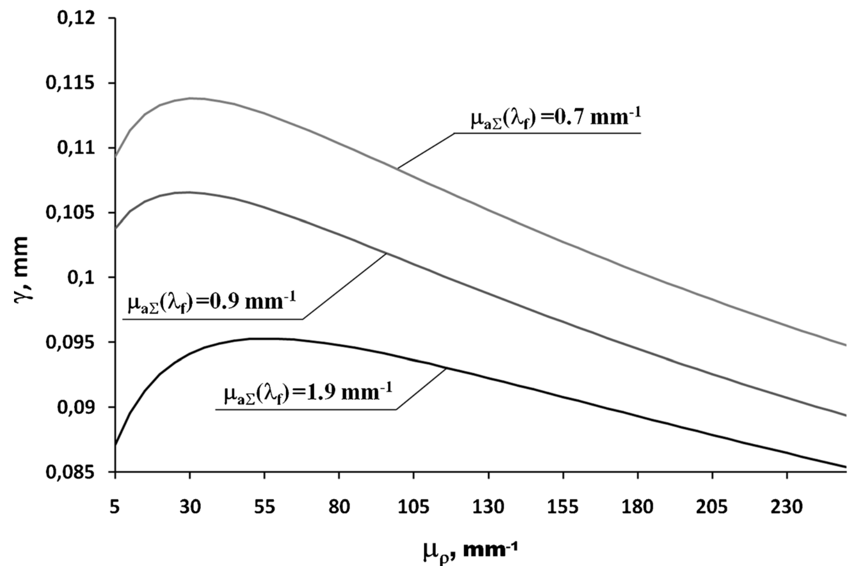

Fig. 7 Parameter γ versus the average density μ_ρ of scatterers in the medium for the case of $\mu_{a\Sigma}(\lambda_e)$=4 mm^{-1}, R(λ_e)=0,03, R(λ_f)=0,02

publications the simplified linear approach to describe the fluorescent signal still exists. For example, the classic handbook by Parker [19] describes the fluorescent intensity I(x) in the point x=H [mm] inside the medium as the well-known simplest function:

$$I(x = H) = 2,3\cdot\Phi_0\cdot\varphi(\lambda_e, \lambda_f)\cdot\mu_{af}(\lambda_e)\cdot C_f\cdot H. \quad (9)$$

This Eq. (9) is frequently used in a lot of studies, for instance, by Meahcov and Sandu in their paper [20] with the reference to the Parker's book. And such examples are numerous. As a result, there is a standard opinion that the fluorescence intensity always linearly depends on the concentration C_f for low concentrations (C_f<<1). It is definitely true for non-scattering media, but, as we saw above, becomes often untrue for light-scattering media. In the last case, the dependence is not linear for any levels of fluorophore concentrations due to the multiple light scattering inside the medium. Multiple light scattering causes such an enhanced path-length for all photons in the medium, that transformations similar to H→γ for Eq. (9) should be taken into account (compare Eq. (9) and Eq. (7)). Also the assumption $A_f(\lambda_e)=\mu_{af}(\lambda_e)$ becomes incorrect.

Additionally, we'd like to note that in all our experiments the experimentally registered flux J(0) was non-monotonic. It differs from our theoretical results a little, but in our theoretical approach only 1D model of the medium was used. Moreover, unlike the experimental setup, in our theoretical model the fluorophore location inside the medium was supposed to be uniform, while in light-scattering measures the photosensitizer solutions were compactly located in the cavity. Therefore, such a difference in results is quite explainable.

All these results show a lot of difficulties which physician can meet while analyzing the in vivo LFS data. From physician's point of view, concentrations of fluorophores - in tumorous tissues, for example - are the most important parameters. But having the information of the backscattered flux J(0) only, he/she cannot make a correct decision about the fluorophore content in inspected tissues. He/she will need additional information about the local optical properties of these tissues. That is, analyzing only the fluorescence absolute intensities J(0) of different fluorophores, their concentration cannot be precisely estimated at the moment without additional calculations or additional experimental evaluation of both parameters γ and $A_f(\lambda_e)$.

This is one of the most important additional implications of our results for practice. Existing LFS diagnostic systems are not able yet to evaluate experimentally both γ and $A_f(\lambda_e)$, and due to the great variability of optical parameters of human tissues, both parameters γ and $A_f(\lambda_e)$ may be more or less accurately determined by only means of real time in vivo measurements. Therefore, for correct use of such systems for the experimental determination of fluorophores concentrations in real practice, the existing diagnostic systems need to be improved by adding this option.

Conclusion

The purpose of this work was both experimental and theoretical study of the character of dependencies of measured fluorescence intensities on tissue optical properties as well as on fluorophore concentrations in tissues. As a result, a set of theoretical and experimental data was obtained describing the influence of fluorophores concentration on registered signals in LFS. Both experimental and theoretical results show a good qualitative agreement between each other. All these results show that the dependence of the fluorescence intensity on tissues' optical properties and on the concentrations of

fluorophores in tissues can be both nonlinear and non-monotonic.

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