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Intensity measurements in scattering media

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Abstract

We describe a new method which allows quantitative measurements of fluorescence intensity in highly scattering media. The measurement principle is based on observing the emission from both the fluorophore of interest with a nanosecond decay time and of a reference fluorophore which displays a much longer microsecond lifetime. The reference fluorophore is placed on rather than in the sample to mimic a sensing device with the long lifetime reference held against the skin. The amplitude modulation of the emission is observed using the standard method of frequency-domain (FD) fluorometry. At an intermediate modulation frequency, the modulation is equivalent to the fractional intensity of the nanosecond fluorophore. The method was tested in 0.5% intralipid, which is more highly scattering than skin. Quantitative intensity measurements were obtained for various concentrations of fluorescein in intralipid, and of the pH sensor 6-carboxyfluorescein (6-CF). Low frequency modulation measurements provide a general method for quantitative measurements in the presence of factors which preclude direct intensity measurements.

Keywords

Fluorescence; Frequency-domain; Sensing; Modulation; Intensity

1. Introduction

Fluorescence intensity measurements are widely used in the research laboratory and in analytical and clinical chemistry [1–3]. In recent years, there has been increasing interest in the use of fluorescence for non-invasive sensing [4,5]. The possibility of non-invasive sensing is based on the low absorbance of tissue at red and near-infrared (NIR) wavelengths, and the increasing availability of long wavelength fluorophores [6–8]. However, it is difficult to perform intensity measurements in highly scattering media [9], which has led to interest in the use of time-resolved fluorescence and lifetime-based sensing for non-invasive fluorometry [10–12]. It is now known that fluorescence lifetimes can be measured in the presence of extensive light scattering, and can even be measured through skin [13].

While the use of lifetime measurements solves many problems associated with non-invasive sensing, it is still desirable to develop methods which would allow intensity measurements through skin and in the presence of extensive scattering. In the present report, we describe a

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simple method to measure fluorescence intensities in scattering media. This method is based on measurement of the modulation of the emission when the sample is excited with amplitude modulated light. In our method, the emission of the scattering sample is observed along with the emission of a long lifetime reference fluorophore. The reference fluorophore need not be within the sample, but can be on the surface outside the sample. This geometry is useful for sensing devices in which the long lifetime reference is held against the skin. The light modulation and measurement frequency is chosen so that the emission from the long-lifetime reference is completely demodulated. Under these conditions, the intensity of the fluorophore of interest with a nanosecond decay time is given by the modulation of the total signal. This method can be applied to any sample which displays changes in intensity, or any sensing fluorophore which shows intensity changes due to its response to an analyte of interest. One can imagine this method being used with a hand-held instrument for point-of-care transdermal measurements.

2. Materials and methods

$Ru(bpy)_3Cl_2 \cdot 6H_2O$ was from Aldrich disodium, fluorescein was from Exciton, and 6-carboxyfluorescein (6-CF) was from Eastmanr/Kodak. Intralipid (20%) was obtained from Kabi Vitrum, and diluted 40-fold to 0.5%. Solutions ranging from pH 5.0 to 9.1 were prepared in 50 mm Tris buffer for the 6-CF studies.

Experiments were performed using the geometry shown as an insert in Fig. 1. The intralipid sample was contained in a 2 mm×10 mm cuvette, with the 488 nm excitation incident on the wider 10 mm surface. This surface of the cuvette was covered with a film of polyvinyl alcohol (PVA) which contained the long-lifetime fluorophore $Ru(bpy)_3Cl_2$. The emission was observed from the 2 mm side through a filter which transmitted part of the fluorescein emission and most of the $[Ru(bpy)_3]^{2+}$ emission. The transmission curve of this filter is shown as a dashed line in Fig. 1.

2.1. Instrumentation

Time-resolved luminescence decays were measured in the frequency domain (FD; ISS, Kaola. using an air-cooled cw-Ar^+ laser (Omnichrome, 543, AP. operating at 488.0 nm (80 mW) as the excitation source. The 488 nm output was amplitude modulated using an electro-optic modulator, which provided modulated excitation from 300 kHz to 150 MHz. This FD instrument was comparable to those described previously [14,15]. Rhodamine B in water with a lifetime of 1.68 ns was used as a lifetime reference in the FD experiments [16].

Luminescence decays were analyzed by non-linear least squares procedures described previously [17,18]. Global analysis of FD emission decay data was performed using programs developed at the Center for Fluorescence Spectroscopy. In the global analysis, the lifetimes were the global parameters and the amplitudes were fitted as non-global parameters. This means that the lifetimes were fitted parameters, but the same at all fluorescein concentrations or all pH values for 6-CF. The amplitudes were also fitted parameters, but were allowed to vary at each concentration on pH values. Uncorrected steady-state emission spectra were obtained on a SLM AB-2 fluorimeter.

3. Theory

Intensity decays were measured using the FD method. This method and procedure for data analysis have been described in detail [17–19]. In this method, one measures the phase (ϕ_ω) and modulation (m_ω). of the emission for various values of the light modulation frequency (ω, in rad/s). A number of different models were used to analyze the FD data. Initially, the intensity decays were analyzed in terms of the multi-exponential model,

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \tag{1}$$

where α_i are the pre-exponential factors and τ_i the decay times. The fractional steady state intensity associated with each decay time is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \tag{2}$$

Generally, $\Sigma \alpha_i$ and Σf_i are constrained to be equal to unity.

For the present report, it is instructive to consider the theory for a sample which displays two widely different decay times, the short (τ_S) and long (τ_L) decay times. For a mixture of fluorophores, the phase and modulation can be calculated using

$$N_\omega = \sum f_i m_i \sin\phi_i \tag{3}$$

$$D_\omega = \sum f_i m_i \cos\phi_i \tag{4}$$

where f_i is the fractional intensity, ϕ_i is the phase angle and m_i is the modulation of each fluorophore. If each fluorophore displays a single exponential decay time τ then

$$\tan\phi_\omega = \omega\tau_i \tag{5}$$

$$m_\omega = \left(1 + \omega^2 \tau_i^2\right)^{-1/2}. \tag{6}$$

The phase and modulation at any given frequency ω are given by

$$\tan\phi_\omega = N/D \tag{7}$$

$$m_\omega = \left(N^2 + D^2\right)^{1/2} \tag{8}$$

For the case of two decay times (τ_S and τ_L), the values of N and D are given by

$$N = f_S m_S \sin\phi_S + f_L m_L \sin\phi_L \tag{9}$$

$$D = f_S m_S \cos\phi_S + f_L m_L \cos\phi_L \tag{10}$$

The total fractional intensity is normalized $f_S + f_L = 1.0$. If the two lifetimes are very different. One can examine the sample at an intermediate modulation frequency such that the modulation of the short component is unity ($m_S = 1.0$. and the modulation of the long component is near zero ($m_L = 0.0$). In this case

$$N = f_S \sin\phi_S \tag{11}$$

$$D = f_S \cos\phi_S \tag{12}$$

Using Eq. (8), and recalling the $\sin^2\theta + \cos^2\theta = 1.0$, one obtains

$$m = f_S . \tag{13}$$

This is a useful result which indicates that the modulation of the emission is the fractional intensity of the short lifetime component. Hence, one can use the modulation of the emission to measure the fractional fluorescence intensity of the short lifetime fluorophore.

Measurements of nanosecond and subnanosecond lifetimes in scattering media can be affected by the time-dependent migration of photons due to the multiple scattering events. This topic has been described in detail [11,12]. When a pulse of light enters the scattering media, the diffusely scattered light is delayed in time and the pulse is broadened. These effects can be described phenomenologically in terms of a time delay (t_L), a decay time for the light in the tissues (t_D) and a term describing the pulse broadening (Δt) [12]. For a fluorophore which displays a lifetime τ, the phase and modulation measurement in scattering media is given by [12],

$$\phi_\omega^S = \arctan(\omega\tau) + \arctan(\omega t_D) + \omega t_L \tag{14}$$

$$m_\omega^S = \left(1 + \omega^2\tau^2\right)^{-1/2}\left(1 + \omega^2 t_D^2\right)^{-1/2} m_{\Delta t} \tag{15}$$

where

$$m_{\Delta t} = \left(1 + \omega^2 \Delta t^2\right)^{-1/2} \tag{16}$$

Examination of Eqs. 14 and 15 indicate that the effects of the lifetime (τ). and the photon decay time (t_D) are similar. Fortunately, the values of the decay time t_D are modest for all but

the shortest lifetimes. For instance, the value of t_D for a position 4 mm deep in the 1% intralipid is about 140 ps [12]. Hence, for lifetimes 1 ns and longer, use of the values of ϕ_ω^S and m_ω^S provides a good approximation (τ_{app}) to the true lifetime (τ).

$$\phi_\omega^S = \arctan\left(\omega\tau_{app}\right) + \omega t_L \tag{17}$$

$$m_\omega^S = \left(1 + \omega^2\tau_{app}^2\right)^{-1/2} m_{\Delta t} \tag{18}$$

Some of the data were analyzed in terms of this model. Hence, we can expect an apparent lifetime for a fluorophore in scattering media to be 0.1 to 0.2 ns longer than in the absence of light scatter. We note the difference between the true and apparent lifetime depends on the method used to measure the phase and modulation of the excitation light. Importantly, the effects due to time-dependent photon migration occur mostly on the subnanosecond timescale and at frequencies above 100 MHz. Hence, it is not necessary to consider these effects for the low-frequency modulation measurements.

The parameters describing the intensity decays were obtained by non-linear least squares and minimization of χ_R^2,

$$\chi_R^2 = \frac{1}{v}\sum_\omega \left(\frac{\phi_\omega - \phi_{c\omega}}{\delta\phi}\right)^2 + \frac{1}{v}\sum_\omega \left(\frac{m_\omega - m_{c\omega}}{\delta\omega}\right)^2 \tag{19}$$

In this expression, the subscript c indicates calculated values for assumed parameter values. The terms δm and δm are the uncertainties in the phase and modulation data, respectively, and v is the number of degrees of freedom. For the present measurements, we used $\delta\phi = 0.3°$ and δms=.007.

4. Results

4.1. Fluorescein concentrations in intralipid

The possibility of quantitative intensity measurements in scattering media was tested using various concentrations of fluorescein in intralipid. Emission spectra are shown in Fig. 1. The maximum fluorescein concentration is 12 mm. The emission from this concentration of fluorescein can be easily seen even in 0.5% intralipid. As the fluorescein concentration is decreased, the intensity at 520 nm decreases. All the emission spectra shown in Fig. 1 were collected with the external PVA film containing $[Ru(bpy)_3]^{2+}$, which served as the reference fluorophore. The emission of $[Ru(bpy)_3]^{2+}$ occurs near 600 nm, and is visible on the long wavelength side of the fluorescein emission.

We examined the frequency response of fluorescein in 0.5% intralipid, without the long-lifetime reference (Fig. 2). The data could be fit reasonably well to a single decay time of 4.22 ns, but with a somewhat elevated value of $\chi_R^2 = 4.7$. The fit was improved using the

models which included the effects of time-dependent photon migration (Eqs. (14)–(18)). Use of this model resulted in a decrease of χ^2_R to 1.2. While the differences between the two fits in Fig. 2 is not dramatic, our experience with the FD measurements allows us to accept the model resulting in $\chi^2_R = 1.2$. Importantly, the effects due to the scattering media are only significant at frequencies above 100 MHz. Hence, these effects can be neglected in the low-frequency modulation measurements which can be performed near 2 MHz.

We next used the configuration shown in Fig. 1 to test the concept of using the modulation to measure the intensity. Fig. 3 shows FD measurements of the emission due to both fluorescein in intralipid and the long-lifetime reference. These data were analyzed globally using Eqs. (14)–(18) (Table 1). The long-lifetime of 1575 ns is essentially equivalent to that observed for $[Ru(bpy)_3]^{2+}$ alone in the PVA film. The lifetime of 3.89 ns is assigned to fluorescein. The modulation data over the intermediate range of frequencies is shown on an expanded scale in Fig. 4. One notices a region from 0.3 to 8 MHz over which the modulation is nearly independent of the light modulation frequency.

The modulation data at 1.8 MHz were used to prepare a calibration curve for the fluorescein concentration (Fig. 5). This curve suggests that the intensity measurements can be rather accurate. Using the concentration range from 0 to 6 μM, an uncertainly in the modulation of ±0.007 results in a fluorescein concentration uncertainty of ±0.1 μM. Hence, the intensity can be accurate to about one part in 60, which is probably adequate for most clinical applications.

Examination of Fig. 5 reveals the modulation does not decrease linearly as the fluorescein concentration is decreased. This effect can be understood by noting that the modulation is approximately equal to the fractional intensity of the short component,

$$m = \frac{f_S}{f_S + f_L}$$

One has to recall that the total intensity in a FD measurement is always normalized to unity, $f_S + f_L = 1.0$. A simple calculation reveals the origin of the non-linear dependence of the modulation on the total intensity. Suppose the initial intensity of the short component is $f_S = 0.9$, and that $f_L = 0.1$, resulting in a modulation of 0.9. Now suppose the intensity of the fluorescein emission is decreased two-fold. This can be simulated by setting $f_S = 0.45$ and $f_L = 0.1$, these values being appropriate prior to normalization. Hence, the two-fold decrease in fluorescein intensity results in a modulation near 0.81, consistent with the data in Fig. 5.

4.2. pH sensing in intralipid

Another possible use of modulation sensing is for measurement of the intensity of a sensing fluorophore in tissues or through skin. This possibility was examined using 6-CF. Fluorescein and its derivatives are well known to display intensities which depend on pH and dissociation of the carboxyl group [20–22]. The ionized form which exists at pH values above 7.5 is highly fluorescent, and the protonated low pH form is essentially non-fluorescent.

To test the possibility of pH sensing in scattering media, 6-CF was diluted in 0.5% intralipid, and the long-lifetime reference was again on the outer surface of the cuvette. The emission spectra show that the intensity of 6-CF decreases as the pH decreases (Fig. 6; Table 2). The emission from $[Ru(bpy)_3]^{2+}$ in PVA film is centered near 600 nm and its intensity is independent of pH. The frequency responses for this pH sensor (Fig. 7) show that the modulation from 0.5 to 8 MHz is dependent on the pH of the intralipid solution. These modulation data were used to create a calibration curve for pH (Fig. 8). For this particular sensor, modulation values accurate to ±0.007 result in an accuracy of ±0.1 pH units. For clinical applications, pH values are typically accurate to ±0.02 [23–25]. Hence, the accuracy will need to be improved for a clinically useful device. However, we have not yet attempted to optimize the method for such clinical applications.

5. Discussion

What are the advantages of intensity measurements based on modulation? The advantages are similar to those available with lifetime-based sensing. Lifetimes are mostly independent of the total intensity, and changes in probe concentrations do not affect the lifetimes. Similarly, the measured modulation will be independent of the total signal level. Hence, modulation-based intensity measurements can be accurate even if the signal level changes due to flexing of fiber optics or changes in the optical configuration. However, modulation-based intensity measurements require that the relative proportion of the long- and short-lifetime emissions remain the same independent of sample positioning and changes in the analyte concentrations.

We note that the measured intensities will depend on the extent of scattering and absorption by the sample. In other experiments (not shown), we found the measured intensities were only weakly dependent on these factors. In the present experiments, the goal is not to obtain a precisely accurate intensity, but to obtain a simple and reliable method to obtain approximate intensities. Greater precision could be obtained by calibration of the measurements for each individual.

One can imagine simple hand-held instruments for modulation intensity measurements (Fig. 9). It is now known that the optical output of light emitting diodes (LEDs) can be modulated up to 100 MHz [26–29]. LEDs consume little power and can easily be driven by batteries. Hence, the modulation sensor could be a small device held near the skin. The long-lifetime complex can be part of the device, so none of the long-lifetime probe enters the sample or tissue. The high chemical and photochemical stability of the metal-ligand complexes suggests the signal from the long lifetime reference will be constant for long periods of time. Hence, such devices may prove valuable for quantitation of intrinsic and extrinsic fluorophores in tissues.

Acknowledgements

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Abbreviations:

PVA	polyvinyl alcohol
bpy	2,2′-bipyridyl
6-CF	6-carboxyfluorescein
FD	frequency domain

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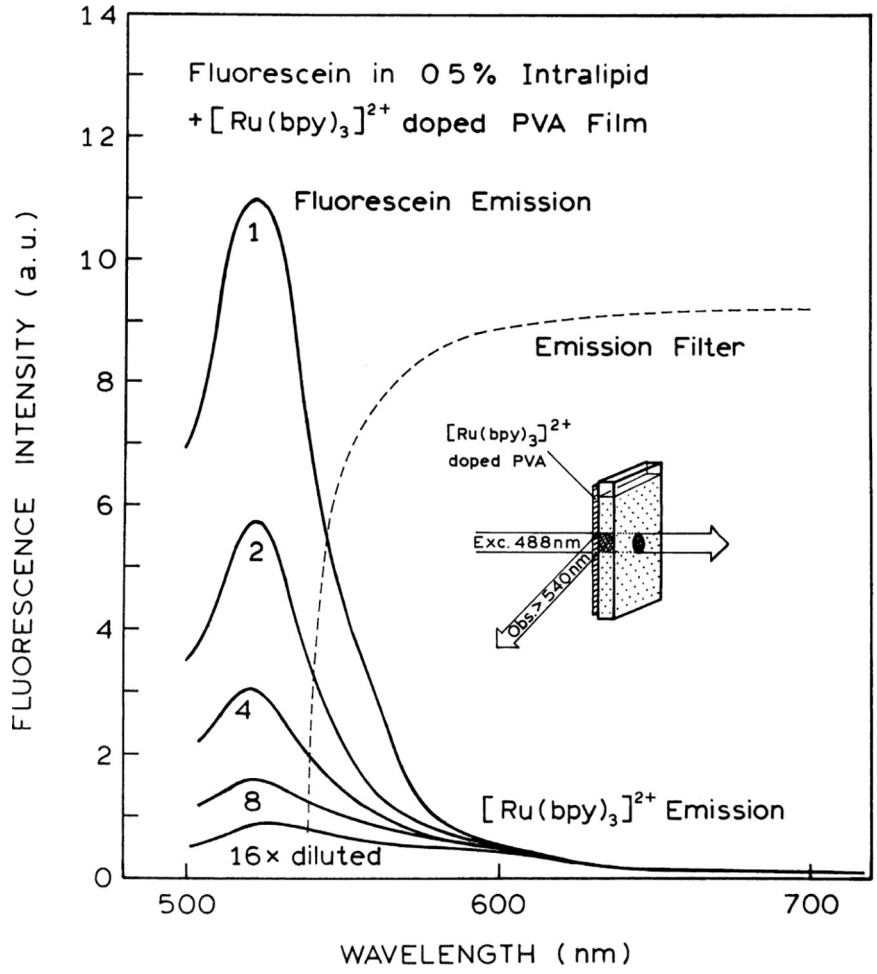

Fig. 1.

Emission spectra of fluorescein in 0.5% intralipid. Emission for $[Ru(bpy]_3]^{2+}$ in the PVA film is responsible for the weak emission near 600 nm.

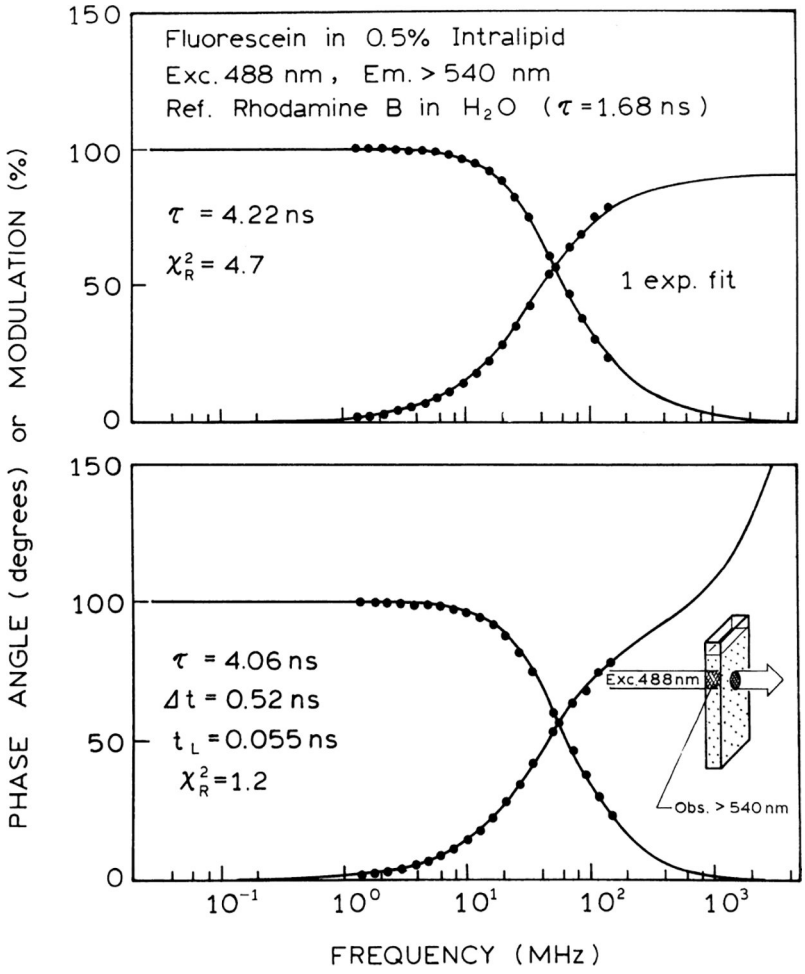

Fig. 2.

FD intensity decay of the intralipid sample containing fluorescein. The cuvette did not have the external $[Ru(bpy)_3]^{2+}$–PVA film.

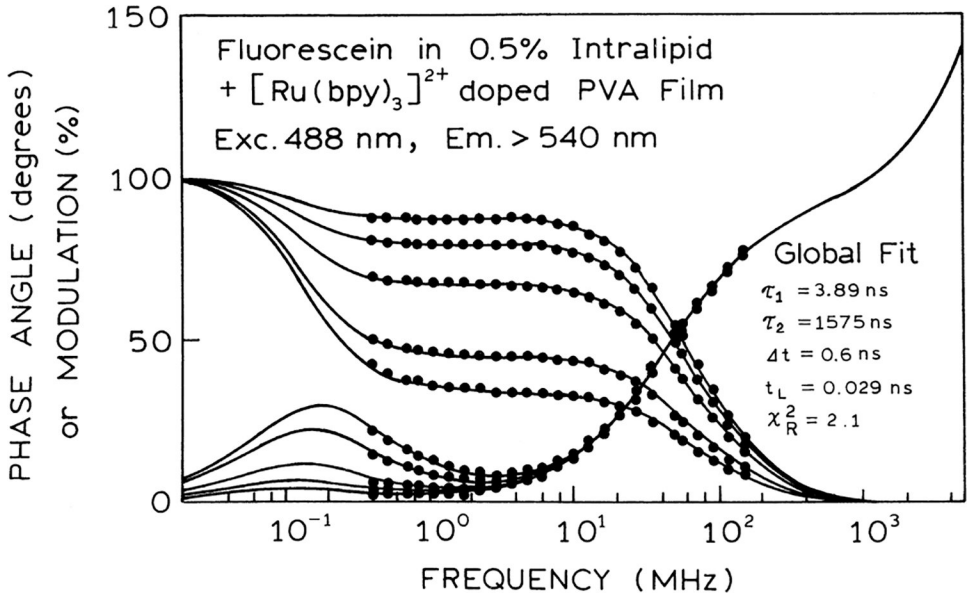

Fig. 3.

Frequency response of the fluorescein–$[Ru(bpy)_3]^{2+}$ intralipid sample shown in Fig. 1.

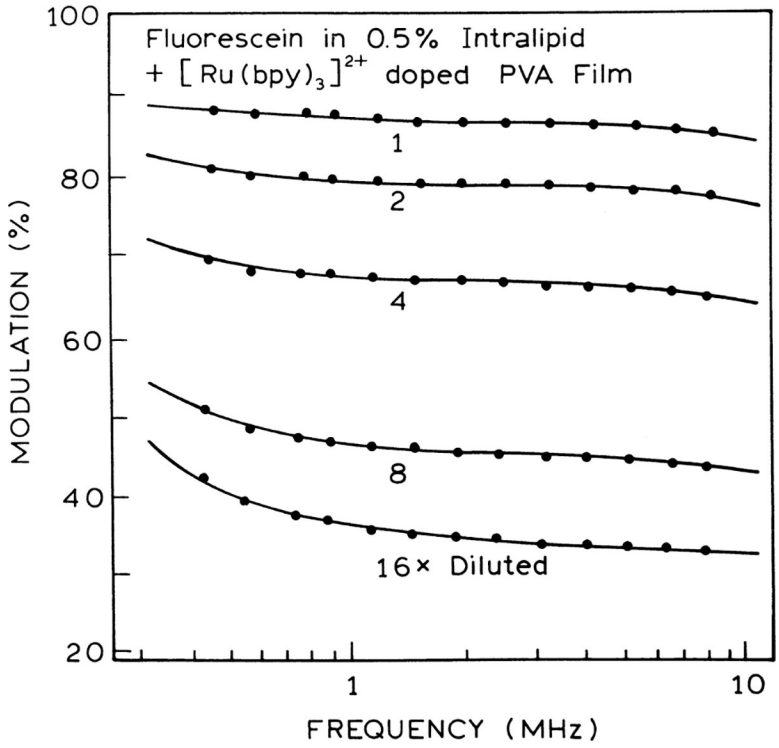

Fig. 4.

Low frequency modulation of the fluorescein–$[Ru(bpy)_3]^{2+}$ intralipid sample shown in Fig. 1.

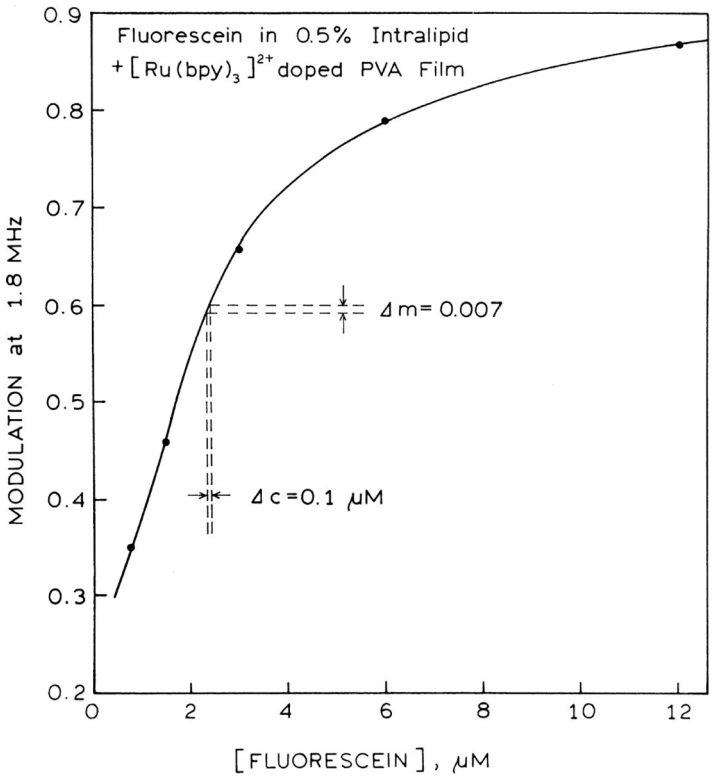

Fig. 5.
Modulation vs. fluorescein concentration.

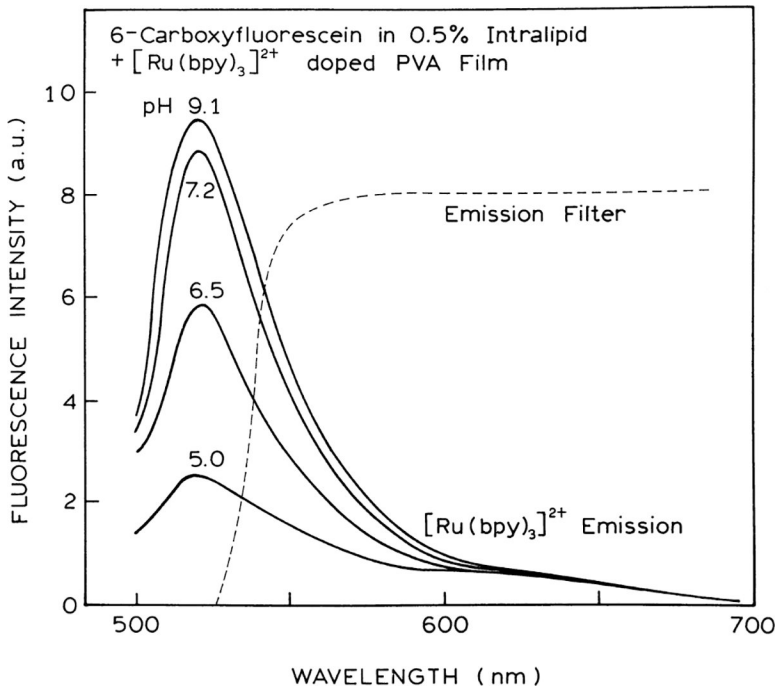

Fig. 6.

Emission spectra of 6-CF in 0.5% intralipid. The experimental arrangement is similar to that shown in Fig. 1, including the long-lifetime standard.

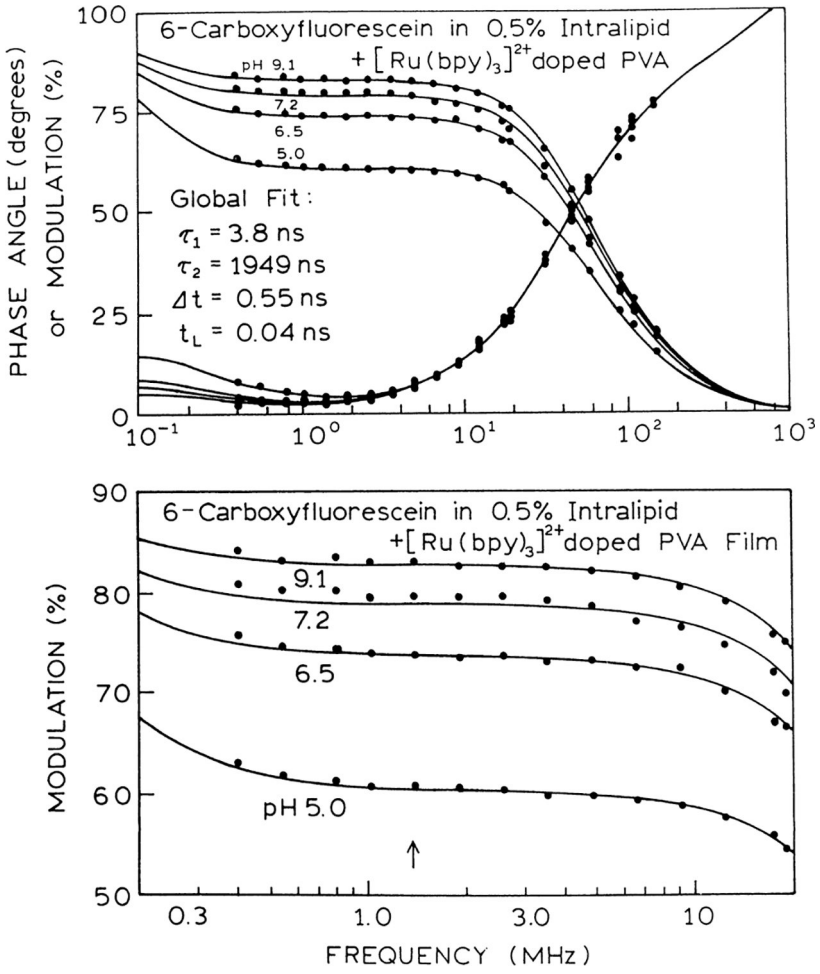

Fig. 7.

Frequency response of the 6-CF–$[Ru(bpy)_3]^{2+}$ intralipid sample shown in Fig. 6.

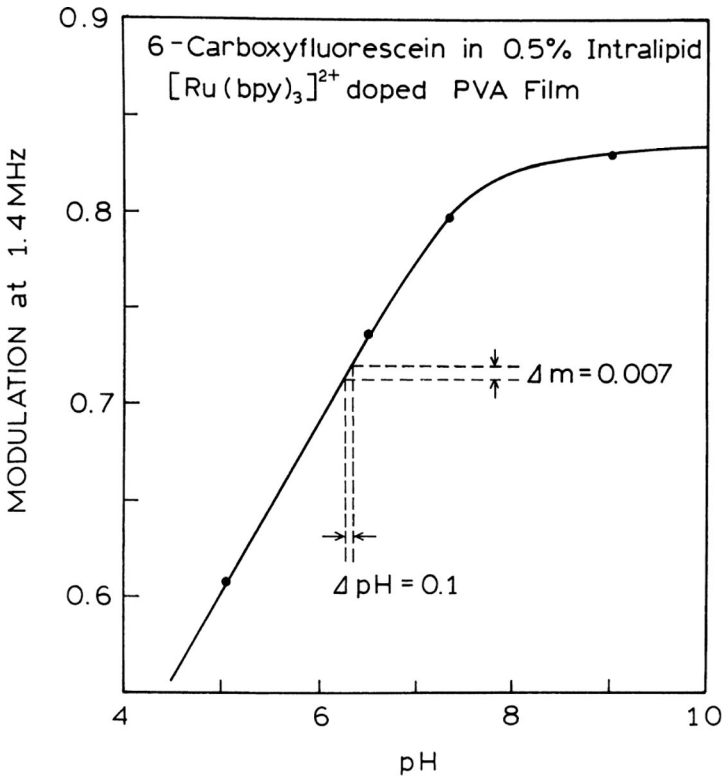

Fig. 8.
Modulation vs. pH calibration curve for 6-CF in 0.5% intralipid.

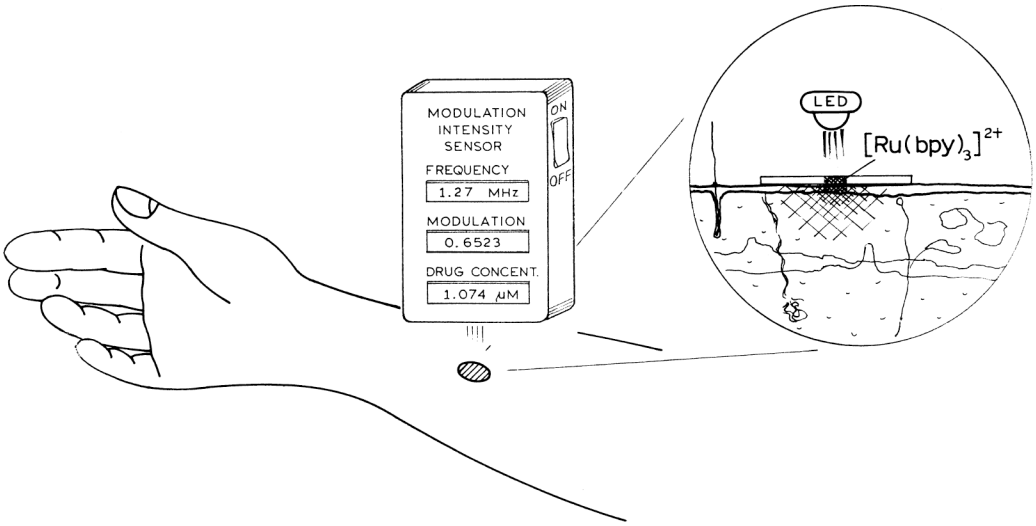

Fig. 9.
Schematic of a portable modulation intensity sensor.

Table 1

Global intensity decay analysis of fluorescein in 0.5% intralipid-$[Ru(byp)_3]^{2+}$-doped PVA film system

Concentration (μm)	τ_1 = 3.89 ns		τ_2 = 1575 ns		Δt (ns)	τ_D (ns)	χ^2_R
	α_1	f_1	α_2	f_2			
12	0.9996	0.871	0.0004	0.129	0.60	0.03	2.1
6	0.9994	0.792	0.0006	0.208			
3	0.9998	0.668	0.0012	0.332			
1.5	0.9970	0.449	0.0030	0.551			
0.75	0.9950	0.338	0.0050	0.662			

Table 2

Global intensity decay analysis of pH sensor in 0.5% intralipid

pH	τ_1 = 3.79 ns		τ_2 = 1949 ns		Δt (ns)	τ_D (ns)	χ^2_R
	α_i	f_i	α_2	f_2			
9.1	0.9996	0.827	0.0004	0.173	0.55	0.04	5.4
7.2	0.9995	0.788	0.0005	0.212			
6.5	0.9993	0.735	0.0007	0.265			
5.0	0.9990	0.602	0.0010	0.398			