FLUORESCENCE NONLINEARITY OF WATER DISSOLVED FLUORESCEIN UNDER THE ACTION OF LASER RADIATION OF HIGH POWER DENSITY

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The nonlinear dependence of the fluorescence of water dissolved fluorescein on a high power density of laser radiation (Φ) with a dye concentration of 10^{-6}—10^{-9} M has been investigated by a pulsed laser microfluorimeter. When measuring the saturation factor Γ as a function of (Φ), one of the methods employed is the determination of true fluorescence intensity (I_t) from the intensity measured (I_m) by a microfluorimeter, and another method is proposed for calculating the excited state lifetime from the function $I_m(\Phi)$.

Introduction

Although lasers of high power and their application in the field of nonlinear optics is well known, the aim of these applications (high resolution laser spectroscopy, SHG, induced Raman scattering, *etc.*) have been first and foremost to investigate atoms and simple molecules and not complicated compounds.

The combined organic compounds (*e.g.* dyes, with broad and strong absorption bands in the visible region — $\sigma_a = 10^{-16}$ cm^2 — and having the short lifetime of the excited state in solution — $\tau = 2$—6 ns), require very high photon flux density (Φ) for fluorescence nonlinearity, a phenomenon mentioned in the theory of saturation of the excited electronic state of a fast three-level system, where the saturation photon irradiance (Φ_s) is defined as [1, 2]:

$$\Phi_s = \frac{1}{\sigma_a} \cdot \frac{1}{\tau} \tag{1}$$

The putting of dye lasers pumped by solid state or gas lasers (*e.g* second or third harmonic of Nd and Rb lasers, or N_2 and excimer lasers) to practical use has not stimulated as yet the investigation into the nonlinear behaviour of the fluorescence of dyes depending on photon irradiance, because Φ of the pumping lasers is quite near the saturation limit, at every exciting wavelength.

But recently fluorescence microscopy in biology and medicine (*i.e.* laser microscopy — laser microfluorimetry [3, 4]) has used tunable pulsed dye lasers as pumping sources. In these experimental cases, when dyes are using several biological macromolecules for labelling, the power density exciting these dyes can change from

10 MW/cm² to 1 GW/cm² — *i.e.* 10^{25}—10^{27} photon/cm s¹. (*E.g.* if the power of the pulse is not more than 5 kW, and the laser beam divergence is 1 mrad, when using a lens of 100 mm focal length (f_1), the diameter of the beam waist (d_1) is 100 μm and the power density (Φ_1) is 50 MW/cm², but when using a $f_2=10$ mm lens, $d_2=$ $=10$ μm and $\Phi_2=5$ GW/cm²).

Investigation into the nonlinear behaviour of the fluorescence of combined organic molecules as a function of high power density has not made great headway yet far-reaching, either theoretically or experimentally [5, 6]. For this reason, we aimed at studying the fluorescence nonlinearity of water dissolved fluorescein using a pulsed laser microfluorimeter, when the power density of the exciting laser light was about Φ_s.

Material and methods

For the object of investigation fluorescein dye (pH = 12) dissolved in water was decided upon. Our choice fell on this dye because the fluorescein had been investigated with conventional methods many times and even by a pulsed laser fluorimeter [7] a few times, and this dye is one of the most popular labelling dyes in biological and medical microscopic investigation [8].

Fluorescein was purified by chromatography, and the solvent was deionized water. The watering was made from a concentration of 10^{-4} M drawn on the tenth part in every case, and the concentrations used were 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M. We applied these low concentrations, because the optical density of the solutions (OD) was very low, and so the power density showed a negligible change through a pathlength of 1 cm. In terms of the Beer—Lambert law a less than 10 *p. c.* absorptivity is after 1 cm pathlength, if the concentration (c) with fluorescein at exciting wavelength of 480 mm (λ_e) is:

$$\mathrm{OD} = \log\frac{I_0}{I} = \varepsilon(\lambda_e)\cdot c\cdot d = \log 1.1, \quad c = \frac{\log 1.1}{\varepsilon(\lambda_e)\cdot d} = 10^{-6}\,\mathrm{M} \tag{2}$$

The experimental set-up

The main parts of the pulsed laser microfluorimeter are shown in Fig. 1.: Nitrogen laser (NL) (built in our institute) with triggering pulse generator, dye laser (DL) (also built in our institute), delaying pulse generator (D), sample holder (S), monochromator (M) with stepping motor, photomultiplier (Ph) with high voltage supply, multichannel analyser (MA), computer (Comp.) $x-y$ plotter ($x-y$), F_1, F_2 neutral optical density filters and L_1, L_2 lenses.

The advantages of this apparatus comes from the use of the pulse measurement technique. The pulse generator of NL triggers everything. This pulse switches the discharge of NL, makes step through D, the stepping motor of M (*i.e.* tunes the monochromator), and opens an electric gate its time-duration is 100—500 ns. The amplitude/digital converter A/D of MA integrates the photocurrent receiving it under measure time, and converting it into a digital signal, which will be accumulated in one of the 1024 channels. MA, after this process makes step on M and is ready to receive the following data, and the first channel follows the last one. So this apparatus

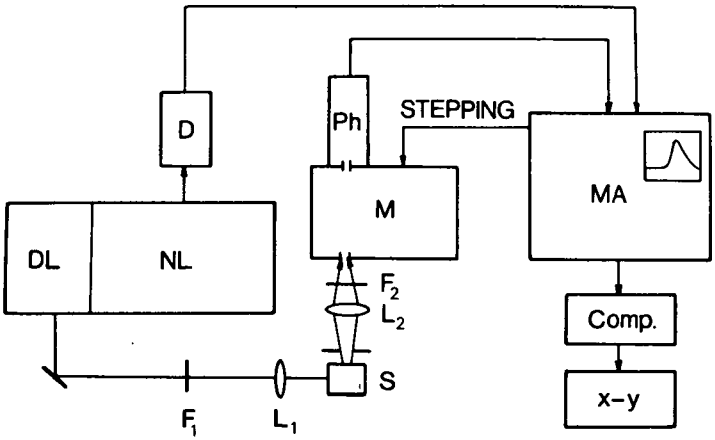

Fig. 1. Pulsed laser microfluorimeter design. NL — nitrogen laser, DL — dye laser, D — delaying pulsed generator, S — sample holder, M — monochromator, Ph — photomultiplier, MA — multichannel analyser, Comp. — computer, $x-y$ — $x-y$ plotter, F_1, F_2 — neutral optical density filters, L_1, L_2 — lenses.

— according to the programme — can measure many times the total spectrum (340 nm—640 nm), and therefore the signal to noise (S/N) ratio gets better. There is still a possibility to integrate the various parts of the full spectrum and to subtract the background signal from the total.

In the dye laser a 4-diethylamino-7-methylcoumarine dye was used. The energy and the duration of the pulses were 15 μJ and 3 ns, respectively, when the repetition rate was 25 Hz and the wavelength was 480 nm. The beam divergence was 1 mrad and the polarization degree on the horizontal plane was 0.99.

We determined the transmission function of the monochromator (Czerny—Turner construction), containing a grate of 600/mm and two concave mirrors of 25 cm, and of the photomultiplier (typed FEU—100), so we got corrected spectra by the $x-y$ plotter.

The linearity of the Ph was studied with respect to the full spectrum of the fluorescein. There was such a concentration ($\sim 10^{-7}$ M) in the cuvette as it give a strong signal. We filtered the fluorescence signal by filters F_2 and we measured photocurrent (I) (I means the digital signal, the bit of the current), concerning unit gain one running. This caracteristic can be seen in Fig. 2. in logarithmic scale (OD is the optical density of the filters F_2).

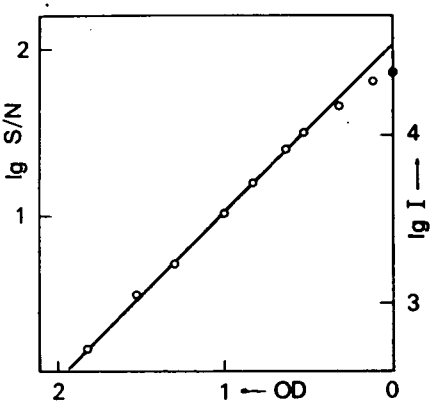

Fig. 2. The dependence of the measured photocurrent of the pohotomultiplier (lg I) and the signal to noise ratio (lg S/N) on the total fluorescence light intensity (OD).

It can be seen in Fig. 2. that if I is 10^4 bit, then undisturbance spectrum can be measured, and the S/N ratio is about 30.

The stereoscopic set-up of the sample holder is shown in Fig. 3.

We have decided on 140 mm for the focal length of lens L_1. The product of this length and the beam divergence are 140 μm, *i.e.* the diameter of the beam waist. In this case, the power density is 25 MW/cm² ($5 \cdot 10^{25}$ photon/cm² s¹), since the average power of the laser pulse is 5 kW, and this value Φ is about 50 times higher, than the Φ_s producing saturation of the excited state ($\Phi_s = 0.5$ MW/cm²).

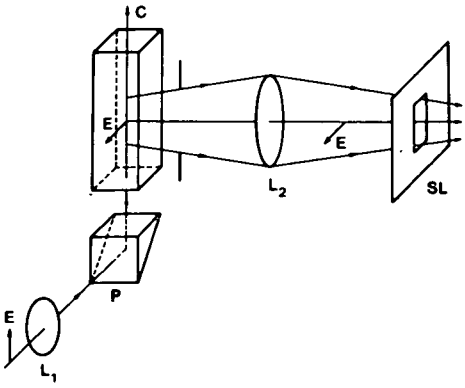

Fig. 3. The stereoscopic set-up of the sample holder. L_1 — lens of 140 mm, P — prism, C — cuvette.

The exciting, focused laser beam passes through prism (P) and goes vertically to the lower part of cuvette (C). The excited volume in the solution is a cylindrical shape, it is parallel to the slit of monochromator (SL), and it is projected in a ratio 1:1 by lens L_2 on to S. The width of the slit was 0.2 mm, so the spectral resolution of M was 2 nm. Vector E is the electric polarization vector of the laser beam, and its direction is unchanged — as compared to the direction of detection — to the set-ups of other investigations concerning the Raman scattering of water [8, 9]. The point, where the beam goes to the cuvette, is not projected on to SL, so the scattered light on quartz is not disturbed.

We made choice of an exciting wavelength of 480 nm, because the fluorescein is highly absorbent at this wavelength, but the other organic traces in the water were less excited. This wavelength, as scattered light, has not disturbed the measurement of the fluorescence spectrum of fluorescein, and the band of the Raman scattering of water ($\sim 3400\ cm^{-1}$) arises at 745—750 nm, when $\lambda_e = 480$ nm.

So the Raman band of the water and the maximum of the fluorescence of the fluorescein are at separated wavelength and the Raman scattering as a solvent blank is not much disturbed only at low concentrations, we measured the total spectra from 340 nm to 640 nm at every concentration and at every power density, five times. The blank signal from the total was subtracted and corrected with the transmission function of the apparatus. Then we calculated the integral of the corrected fluorescence spectra (I).

In every case we started the mesurement of function $I_m(\Phi)$ without filters F_1 and we put before the monochromator filters F_2 of such optical density that the photomultiplier could get a light intensity corresponding to 10^4 bit. Then increasing the OD of F_1 we decreased the OD of F_2 at the same time.

Results

Fig. 4. shows the corrected spectra with the transmission function of the detector of 0.01 N water-dissolved fluorescein of 10^{-9} M (λ_e=480 nm and the observation of the radiation was perpendicular to the laser beam and as well as to E vector). The power density of the exciting laser light was filtered at various spectra what is shown in the Table I.

We can see from Fig. 4., when that we filter the power density, the maximum intensity of the Raman band of water is changing simultaneously with Φ, but the flouresence intensity is not, and when the Φ is only 2—3% of the beginning power density — *i.e.* $\sim 10^{24}$ photon/cm^2 s^1 — the fluorescence intensity compared to the intensity of Raman band shows no change.

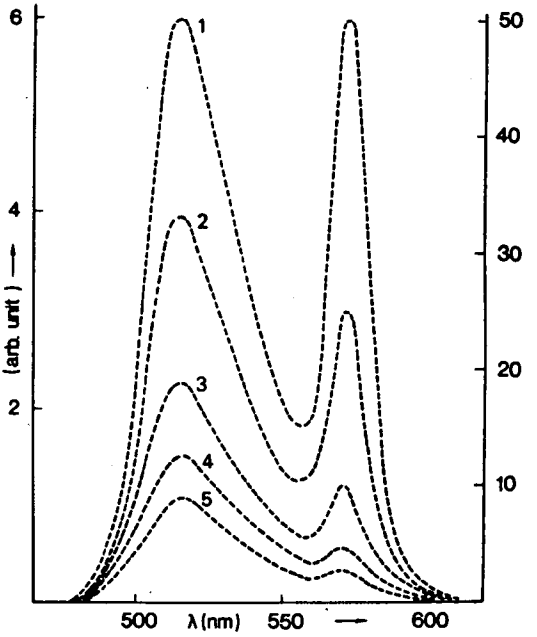

Fig. 4. The spectra of the total scattered light fluorescence, Raman scattering of the 10^{-9} M fluorescein in water (pH=12) as a function of the several exciting power density Φ. 1—25 MW/cm^2, 2—12.5 MW/cm^2, 3—4.16 MW/cm^2, 4—1.25 MW/cm^2, 5—0.8 MW/cm^2.

As a second step, we also measured the dependence of the total fluorescence intensity (I) on the power density of the exciting light with other concentrations (10^{-8}, 10^{-7}, 10^{-6} M). This connection is shown in Fig. 5 in log-log scale. In Fig. 5 small cirdes are the symbols of the experimental results. I depends linearly on Φ, if the photon flux density is lower than 10^{24} cm^{-2}s^{-1}. So to most of the measured points at a 10^{-6} M concentration a straight line at angles up to 45° — expressive of the linear connection — can be fitted, and we lengthened this line to the vertical axis. We have drawn three further theoretical lines at 10^{-7}, 10^{-8}, 10^{-9} M parallel to the line concerning 10^{-6} M. These lines were shifted with one, two and three decades. One part of the measured points concerning 10^{-7} M was perfectly congruent with the

Table I

Curve number	1	2	3	4	5
OD of filters	0	0.3	0.8	1.3	1.5
Filtering (%)	100	50	16.6	5	3.3
Power density (MW/cm^2)	25	12.5	4.2	1.25	0.84
Photon flux density (photon/cm^{2}s^1)	$5\cdot10^{25}$	$2.5\cdot19^{25}$	$8\cdot10^{24}$	$2.5\cdot10^{24}$	$1.6\cdot10^{24}$

theoretical line, while the linear part, at lower concentrations could not be measured because of a significant decrease in the fluorescence detected.

The measured points concerning an Φ higher, than $10^{24}\,\mathrm{cm^{-2}\,s^{-1}}$ can be found on the log-log scale, also along a straight line (broken line). The slope of these lines significantly deviates from the line angles to 45°, and the tangent of this angle is equal to 0.55, *i.e.* about 0.5. Such a kind of line on the log-log scale is a parabola on the linear-linear scale:

$$I_m \sim \sqrt{\Phi}, \quad \text{if} \quad 3 \cdot 10^{24}\,\mathrm{cm^{-2}\,s^{-1}} < \Phi < 50 \cdot 10^{24}\,\mathrm{cm^{-2}\,s^{-1}} \tag{3}$$

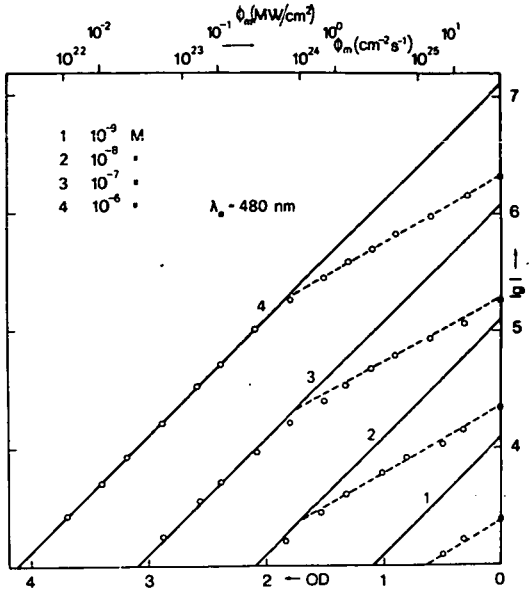

Fig. 5. The dependence of the fluorescence intensity (I) of the water dissolved fluorescein on the power density of the exciting light pulses Φ with 10^{-9} M (1), 10^{-8} M (2), 10^{-7} M (3), 10^{-6} M (4) concentrations. Exciting wavelength λ_e=480 nm. Continuous line — linear part, broken line — non-linear part.

We can draw some important conclusions from Fig. 5: The broken lines intersect the lines of 45° at the same Φ' ($\sim 10^{24}\,\mathrm{cm^{-2}\,s^{-1}}$), their slope is also the same and they are equidistant (this separation is exactly 1.0). The nonlinear behaviour of the fluorescence of fluorescein in water solution at every concentration occurs at the same Φ' value, *i. e.* Φ' depends only on the molecular parameters corresponding to Eq. (1), so that *this phenomenon is the saturation of the excited state.*

But the approximation given in Eq. (3) does not show saturation. Equal to the former effect is that factor Γ (Γ is the ratio of I^0 — the number of fluorescent photons, which would have been detected in the abscence of saturation, the value of the linear angles to 45°, — and of I_m — the measured number of fluorescent photons — at the same excitation) as the function of Φ is not linear.

Conclusions

Experimental results shown in Fig. 5 can be used in many respects: to several purposes.

1. One of the possibilities is the calculation of the true fluorescence intensity I^0 at a Φ value, from the measured intensity with the help of Γ, *i.e. the measured fluorescence intensity is needed for corrections made in terms of the saturation factor.* For the purposes of correction measuring function $I_m(\Phi)$ at an optional concentration is quite sufficient. (It is best to carry out at the concentration limited by Eq. (2), because the longest linear part of function $I_m(\Phi)$ can be observed in this case). *E.g.* in our measurements the photon flux density without filtering is $5 \cdot 10^{25}\,\mathrm{cm^{-2}\,s^{-1}}$ and the $\lg \Gamma = 0.8$. It means that if is with the I_m produced with 6.3 that we obtain the true fluorescence intensity. It is an important factor which has to be taken into account, if we want quantitative analysis from fluorescence measurement under microscope.

2. The other possibility is *the determination of the lifetime of the excited state at very low concentrations* 10^{-6}—10^{-8} *M*. This determination is based upon the observation that two straight lines, which are fitted to the measured points, intersect in the log-log scale about $10^{24}\,\mathrm{cm^{-2}\,s^{-1}}$ with accuracy ± 0.1. Using this value together with the value of the absorption cross-section at 480 nm $\sigma_a = 2.2 \cdot 10^{-16}\,\mathrm{cm^2}$ we can calculate from Eq. (1) τ, which is given as 4.5 ± 1.0 ns. It is in good agreement with the results obtained by other methods at low concentrations [10—12].

3. The third possibility is *the calculation of the absorption cross-section* from Eq. (1), if we use a tunable dye laser, and if its power density is known at every wavelength, and if the lifetime of the excited state of the molecule studied is measured by an other method *at low concentrations* 10^{-6}—10^{-8} *M*.

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ФЛУОРЕСЦЕНТНАЯ НЕЛИНЕЙНОСТЬ ФЛУОРЕСЦЕИНА, РАСТВОРЕННОГО В ВОДЕ, ПРИ ВОЗБУЖДЕНИИ ИМПУЛЬСАМИ ЛАЗЕРНОГО ИЗЛУЧЕНИЯ ПРИ ВЫСОКОЙ ПЛОТНОСТИ МОЩНОСТИ

Б. Немет, И. Шанта и Л. Козма

Нелинейная зависимость флуоресценции флуоресцеина, растворенного в воде, исследована импульсным лазерным микрофлуориметром при высокой плотности мощности лазерного излучения (Φ), при малых концентрациях красителя 10^{-6}—10^{-9} моль. На основании измерения зависимости фактора насыщения флуоресценции (Γ) от Φ, разработан метод для расчёта истинной интенсивности флуоресценции (I) из измеренной микрофлуориметром интенсивности (I_M), что является предложением нового метода для определения времени жизни возбуждённого состояния из функции $I_M(\Phi)$.