COUMARIN 6, HYPERICIN, RESORUFINS, AND FLAVINS: SUITABLE CHROMOPHORES FOR FLUORESCENCE CORRELATION SPECTROSCOPY OF BIOLOGICAL MOLECULES

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In this work we show that the dyes coumarin 6, hypericin, 7-*O*-ethylresorufin and resorufin are suitable for fluorescence correlation spectroscopy (FCS) and demonstrate the use of these dyes in physiologically relevant protein studies. Since coumarins are metabolised by cytochromes P450, the binding of coumarin 6 to cytochrome P450 3A4 was investigated by FCS. Coumarin 6 appears to be a very bright non-covalent cytochrome P450 label. When titrating cytochrome P450 3A4 with coumarin 6, the diffusion time of the coumarin 6/cytochrome P450 3A4 complex increases roughly two-fold at protein concentrations higher than 1 μmol l^{-1}, indicating the formation of cytochrome aggregates. FCS of the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) shows that both endogenous dyes undergo photobleaching. Moreover, FAD appears to be present to great extent, as a non-fluorescent intramolecular complex. Analysis of the FCS data of the flavoprotein NADPH-cytochrome P450 oxidoreductase (molecular weight 76 500) yielded two components. While the slow component corresponds to a globular protein with the molecular weight about 75 000, the fast component appears to be due to free diffusing FMN and FAD molecules. The amount of free FMN and FAD increases with increasing laser power. At high laser power a complete photodissociation of FMN and FAD occurs.

Keywords: Fluorescence correlation spectroscopy; Cytochrome P450; NADPH-cytochrome P450 oxidoreductase; Single molecule spectroscopy.

The concepts of fluorescence correlation spectroscopy (FCS) have been developed at the beginning of the 1970s (ref.[1]). The first publications using FCS showed that the analysis of the statistical fluctuation of fluorescence may yield information about translational diffusion[2], rotational motion[3], and chemical kinetics[2]. However, several more recent experimental ad-

vances in single molecule spectroscopy have been necessary (for a list of those advances see, Table I in Maiti *et al.*[4]) to allow for routine and rapid FCS measurements. Considering the current demand for fast and sensitive analytical methods, it is not surprising that at present time the number of applications of this technique is growing very rapidly of present.

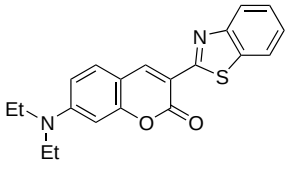
In a FCS experiment, a focused laser beam illuminates a volume element of about 10^{-15} l by using confocal or multiphoton microscopy. The volume is so small that at a nmol l^{-1} concentration at a given time, it may host only one fluorescent particle out of many under analysis. The single fluorescent molecule diffusing through the illuminated volume is detected by an avalanche photodiode and the time-course of the resulting signal is recorded. The autocorrelation function of the time course of the fluorescence signal gives information about the number of molecules in the illuminated volume element and their characteristic translational diffusion time. Since the size of the illuminated volume is known, the concentration and diffusion coefficient of the fluorescent species are determined. In the majority of applications, diffusion properties of two species with different molecular weight are analysed. When a fluorescently labelled low-molecular-weight compound is binding to the high-molecular-weight compound, titration allows for the determination of equilibrium binding constants. This principle can be used for example for the characterisation of interactions between different proteins[5], proteins and membranes[6], or polynucleotides and DNA (ref.[7]). Moreover, chemical or biochemical reactions leading to a marked change in the molecular weight can be analysed in real time[8]. The high spatial resolution of FCS allows for the characterisation of diffusion processes in different compartments of the cell[9]. It should be noted here that, besides the above described translational diffusion, also processes like rotational diffusion, chemical reactions, as well as sample flow can cause fluctuations in the fluorescence signals. The analysis of the statistical fluctuations of fluorescence caused by the above mentioned processes has been reviewed by Thompson[10].

A main limitation in the application of FCS is the requirement for the used dye: a good FCS dye should combine a high fluorescence and a low intersystem quantum yield with high photostability. Thus, being moreover limited by the emission lines of the used laser, the assortment of chromophores for labelling the system of interest is rather small and the applications of FCS using natural chromophores are extremely rare. Table I gives an overview of the used dyes in FCS studies when employing excitation by an Ar^+ laser (488 and 514 nm).

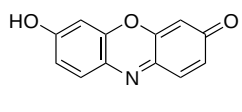
TABLE I
Overview of chromophores used in FCS with excitation by an Ar^+ laser (488 and 514 nm)

Chromophore	Ref.
Rhodamine Green	11
Rhodamine 6G	12
NBD (4-Chloro-7-nitrobenzo[1,2,5]oxadiazole	13
Oregon Green (2′,7′-difluorofluorescein)	14
Bodipy (8-(Bromomethyl)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene)	15
Cy2 (FluoroLink™ Cyanin 2)	16
Alexa Fluor™ 488	17
Ethidiumbromide	18
TMR (Tetramethylrhodamine)	19
Fluorescein	20
GFP (Green fluorescent protein)	21

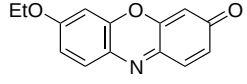
Coumarin 6 [3-(benzothiazo-2-lyl)-7-(diethylamino)-2*H*-chromen-2-one] is a naturally occurring constituent of many plants. Metabolism of coumarin and related compounds was shown to be performed by cytochromes P450 (CYP) in a variety of mammals including human. For example, the 7-hydroxylation of coumarin in human liver by cytochrome 2A6 (CYP2A6) has been studied quite recently[22]. Among cytochromes P450, the



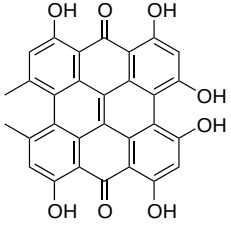
coumarin 6



resorufin



7-*O*-ethylresorufin



hypericin

cytochrome 3A4 (CYP3A4) is known to be the most important for human medicine as it metabolises majority of drugs and other xenobiotics not only in the liver, but also in other tissues[23]. In the liver, it is the most abundant

CYP present representing over one third of the total CYP content. As it ranks among to the relatively less stable CYP enzymes[24] and its enzymology is not simple[25], an understanding of the structure–function relationship of CYP3A4 is still missing. To find an optimum fluorescent probe for CYP enzymes and for CYP3A4 in particular, the substrates of CYP enzymes and their analogs are the compounds of choice[26]. Hypericin (component of St John's wort plant extract, from *Hypericum perforatum*, known for its antiviral and anticancer effect and used in treatment of depression)[27] is a substrate of CYP3A4 (ref.[28]) with known photophysics[29].

Flavins, natural and biological fluorophores, are very important prosthetic groups in flavoproteins. Being ubiquitous, they are involved in many metabolic oxidation-reductions and in electron transport processes in living organisms. Flavins in the excited state play an important role in photobiology. For example, bioluminescent organisms provide a rich source of fluorescent proteins containing flavins[30]. The NADPH: cytochrome P450 (cytochrome c) oxidoreductase has been chosen as a representative of flavoproteins having both, flavin mononucleotide (FMN) and the flavin adenine dinucleotide (FAD) in its molecule. Moreover, its involvement in the electron transporting system of cytochromes P450 makes it an interesting subject for further studies, *e.g.* of interactions of the respective prosthetic groups and electron transfer.

The purpose of this work is two-fold: First, we present coumarin 6, hypericin (1,3,4,6,8,13-hexahydroxy-10,11-dimethylphenanthro[1,10,9,8-*opqra*]-perylene-7,14-dione), 7-*O*-ethylresorufin, resorufin, FMN and FAD as "new" chromophores for fluorescence correlation spectroscopy and, second, we demonstrate what kind of information about the interaction of these endogenous and exogeneous dyes with biologically relevant proteins (cytochrome P450 3A4 and NADPH-cytochrome P450 oxidoreductase) can be obtained with FCS.

EXPERIMENTAL

Fluorescence Correlation Spectroscopy

FCS measurements were carried out using a fluorescence correlation spectrometer "ConfoCor" manufactured by Carl Zeiss Jena. ConfoCor is a Ar^+-laser-adapted AXIOVERT 135 TV microscope with a 100/100 binocular phototubus. The air-cooled Ar^+-laser supplies two excitation wavelengths (488 and 514 nm). Further major components are an *x*–*y*–*z* adjustable pinhole, an avalanche Photodiode SPCM-200-PQ, and a hardware correlator (ALV-5000). The normalized autocorrelation function $G(t)$ for a fluorescence intensity signal $I(t)$ fluctuating around a temporal average

$$I(t) = \langle I(t) \rangle + \delta I(t) \tag{1}$$

is for any delay time given by

$$G(t) = 1 + \langle \delta I(t)\, \delta I(t + \tau) \rangle / \langle I(t) \rangle^2 \,. \tag{2}$$

The brackets denote temporal averages, and δ is the fluctuation symbol.

A general solution for the three-dimensional autocorrelation function $G(t)$ regarding translational diffusion in an ellipsoid confocal volume element yields the following formula for the situation that the fluorescence decay (τ_f) and translational diffusion (τ_d) are well separated in time[31] ($\tau_f << \tau_d$):

$$G(t) = 1 + \frac{1}{N} \frac{1}{1 + (t/\tau_D)} \left\{ \frac{1}{1 + (t/\tau_D)(\omega_2^2/\omega_1^2)} \right\}^{1/2} , \tag{3}$$

where ω_1 is the radius of the volume element in *xy* plane, ω_2 is the half-length of the volume element in *z* direction and *N* is particle number. When knowing ω_1 and τ_D, the diffusion coefficient *D* can be determined ($\tau_D = \omega_1^2/4D$).

If the dye shows a substantial intersystem-crossing quantum yield, the long-living triplet will be populated and Eq. (*3*) has to be extended to the form[32]:

$$G(t) = 1 + \frac{1}{N[1 - T]} \left[1 - T(1 - e^{-t/t_0}) \right] \frac{1}{1 + (t/\tau_D)} \frac{1}{\sqrt{1 + S^2 (t/\tau_D)}} \,, \tag{4}$$

where *T* is a triplet fraction, t_0 is a triplet time constant and *S* is the structure parameter ($S = \omega_2/\omega_1$).

If two molecules with different diffusion times (or coefficients) τ_i, but the same spectral characteristic (*i.e.*, quantum yield, position and band shape of emission spectrum) are present, Eq. (*4*) has to be extended:

$$G(t) = 1 + \frac{1}{N[1 - T]} \left[1 - T(1 - e^{-t/t_0}) \right] \left[\frac{1 - Y}{1 + (t/\tau_1)} \frac{1}{\sqrt{1 + S^2 (t/\tau_1)}} + \frac{Y}{1 + (t/\tau_2)} \frac{1}{\sqrt{1 + S^2 (t/\tau_2)}} \right] , \tag{5}$$

where $\tau_{1,2}$ are diffusion times of components 1 and 2, respectively, *Y* is a fraction of component 2 of the overall fluorescence signal.

The hardware correlator is directly creating the autocorrelation functions $G(t)$, which were fitted using the underneath defined Eqs (*4*) and/or (*5*) applying the FCS Access Fit software (EVOTEC BioSystems, Hamburg, Germany).

All presented data evaluations have been performed by fixing the structure parameter, which was obtained together with the confocal volume by measurements of rhodamine 6G solution using a one-component fit. In order to preserve the experimental condition, characterisations of fluorescence dyes (Table II) was carried out in one sequence. In the data evaluation of the NADPH-cytochrome P450 oxidoreductase as well as in cytochrome P450 titration experiments, a two-component model has been used. In the coumarin 6/CYP3A4 experiment, the data evaluation was performed by fixing the fast component τ_1. The τ_1 was

obtained by measuring a 5 nM solution of coumarin 6 using a one-component fit. In the latter experiments the diffusion times of both component have been determined by free fits. The resulting diffusion times are expressed as the mean ± standard deviation of the mean value.

Materials and Sample Preparation

FMN was purchased from Fluka. Coumarin 6, resorufin, FAD, and NADP were obtained from Sigma. Hypericin was a gift of Dr P. Hodek (Charles University, Prague). All chemicals were reagent grade or better.

Cytochrome P450 3A4 used was a human recombinant enzyme obtained from membranes of transformed *Escherichia coli* cells by a procedure based on method of Guengerich[33]. Adsorption chromatography on hydroxyapatite was used to repurify the enzyme and to concentrate the samples.

The basic idea behind the preparation procedure of NADPH-cytochrome P450 oxidoreductase is identical to the one published[34]. However, the exact procedure[35] differ from the one suggested[34] (for details see ref.[35]), especially in terms of the used buffers, chromatographic columns or solubilisation detergent. All presented experiments were carried out in presence of 50 mM Tris buffer, unless stated otherwise.

TABLE II
Characterisation of fluorescent dyes used in FCS measurement in water (concentration 1 nmol l^{-1}, excitation laser power was 1mW)

	M_r	Diffusion time µs	Triplet fraction %	Excitation wavelength, nm	Diffusion constant 10^{-10} m^2 s^{-1}	Fluorescence quantum yield	Counts per molecule
Rhodamine 6G[a]	479.02	38 ± 2	7.5	488	2.8[b]	0.94[c]	72
Coumarin 6[a]	350.44	29 ± 1	14.4	488	3.6	0.92[d]	50
7-*O*-Ethylresorufin[a]	241.25	33 ± 3	15.0	488	3.2	0.23[d]	16
Rhodamine 6G[e]	479.02	58 ± 3	8.7	514	2.8[b]	0.71[d]	62
Hypericin[e]	504.45	53 ± 5	17.9	514	3.1	0.40[f]	6
Resorufin[e]	235.18	41 ± 2	37.0	514	4.0	0.21[d]	12

[a] Detection volume element $V = 0.36 \cdot 10^{-15}$ l; [b] ref.[17]; [c] ref.[36]; [d] determined by method of the Parker and Rees[37] in ethanol ($c \approx 1$ µmol l^{-1}), using Rhodamine 6G as the reference compound; [e] $V = 0.58 \cdot 10^{-15}$ l; [f] ref.[38].

RESULTS AND DISCUSSION

New Exogenous Dyes for FCS: Coumarin 6, Resorufin, 7-O-Ethylresorufin, and Hypericin

To our knowledge, the four presented dyes, coumarin 6, resorufin, 7-*O*-ethylresorufin, and hypericin, have not been published as dyes in FCS measurements. The first three dyes are prototypic substrates of the CYP enzymes. Hypericin has been shown to be a substrate of CYP3A4 (ref.[29]). Table II summarises some of the properties determined by FCS.

In terms of photophysical requirements, like fluorescence and intersystem quantum yield, coumarin 6 appears to be comparable with the FCS standard dye, rhodamine 6G. In this respect, resorufin and hypericin are inferior when compared with coumarin 6 and rhodamine 6G. Considering standard deviations of about 10% of the determined diffusion times, it appears difficult to clearly distinguish by FCS between diffusion properties of the investigated low-molecular-weight compounds.

Interaction Between Coumarin 6 and Cytochrome P450 3A4 (CYP3A4)

A physiologically relevant task of this work has been the characterisation of the binding behaviour of coumarin 6 (τ_1 = 31 μs) to cytochrome P450 3A4 (CYP3A4). Titration with CYP3A4 of a low concentration (up to 1 μmol l^{-1}) led to the appearance of a second diffusion component with a diffusion time τ_2 of 0.64 ms (for illustration, see Fig. 1). Using the same experimental conditions, the FCS analysis with rhodamine 6G and coumarin 6 in buffer yielded diffusion times of 38 and 31 μs, respectively. From diffusion coefficients of $2.8 \cdot 10^{-10}$ m^2 s^{-1} for rhodamine 6G and from structure parameter S = 5.4, the diffusion coefficient of $3.5 \cdot 10^{-10}$ m^2 s^{-1} for coumarin 6 was calculated. Assuming globular (spherical) shape of this protein with a molecular weight[39] of 57 000 (502 amino acids), the diffusion coefficient is supposed to be around $7.5 \cdot 10^{-11}$ m^2 s^{-1}, corresponding to a diffusion time of 0.15 ms under the used experimental conditions. Note that deviations from the globular (spherical) shape of the protein result in much smaller diffusion coefficients, and thus in much larger diffusion times. When considering a hypothetical case of a protein of 502 amino acids existing exclusively in the α-helical form and having the shape of a "perfect" rod, the diffusion coefficient is supposed to be $1.17 \cdot 10^{-11}$ m^2 s^{-1}, corresponding to a diffusion time 1.9 ms. The estimated diffusion time of 0.64 ms corresponds to diffusion coefficient of $1.63 \cdot 10^{-11}$ m^2 s^{-1}. This observation indicates that

the shape of the CYP3A4 is deviating significantly from a spherical shape. This conclusion corresponds well to the information on the shape of another cytochrome 450 (CYP2C5). This CYP2C5 is the only microsomal liver CYP enzyme, which has been recently successfully crystallised[40]; the shape of its molecule is not spherical, resembling more an ellipsoid than a rod or a sphere.

At CYP3A4 concentration higher than 1 μmol l^{-1} the diffusion time of the second component markedly increased (Fig. 2). As shown in Fig. 2, the diffusion time τ_2 changed from approximately 0.64 to 1.1 ms when titrating up to a CYP3A4 concentration higher than 1 μmol l^{-1}. Under the given experimental conditions, the diffusion times of 0.64 and 1.1 ms correspond to diffusion coefficients of $1.63 \cdot 10^{-11}$ and $9.42 \cdot 10^{-12}$ m^2 s^{-1}, respectively. Apparently, CYP3A4 aggregates at concentrations higher than 1 μmol l^{-1}. For this step it would be desirable to deduce the aggregation number from the roughly two-fold increase in the diffusion time. However, since the diffusion time is strongly dependent on the shape of the aggregates, we can only roughly estimate the size of these CYP3A4 aggregates. When basing the calculation of the aggregation number on the apparent by wrong assumption of globular monomers and CYP3A4 aggregates, the increase in the diffusion time from 0.64 to 1.1 ms diffusion time results in an aggregation number of six. It is worth mentioning that the existence of CYP3A4 hexamers has been demonstrated for other liver P450 enzymes. The microsomal CYP2B6

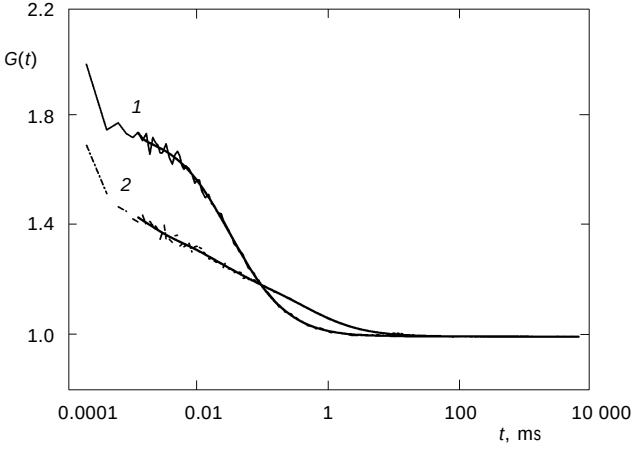


FIG. 1

Autocorrelation functions of free coumarin 6 (τ_1 = 31 μs; 5 nmol l^{-1}, (*1*)) and cytochrome P450 3A4 (0.33 mmol l^{-1})/coumarin 6 (τ_2 = 640 μs; 5 nmol l^{-1}) mixture (*2*)

(also labelled as LM2) as well as a soluble bacterial CYP101 (also labelled as P450cam) have been shown to form hexamers[40,41].

Another interesting observation in the titration experiments was that the aggregation of CYP3A4 prevented coumarin 6 of being almost entirely bound to the protein. We tried to prevent the aggregation by increasing the ionic strength of the buffer (300 mM K_2HPO_4; pH 7.5 instead of water in previous experiments) or by adding ethanol in concentration of 200 mmol l^{-1}. The first attempt did not change the aggregation profile. Ethanol prevented aggregation of cytochrome P450 3A4 and allowed for protein titration up to almost full coumarin 6 binding (see Figs 2, 3).

Fluorescence Correlation Spectroscopy of the Endogenous Dyes FMN and FAD

The numbers of fluorescence photons, which are emitted by flavins, are considerably lower compared to the used rhodamine dyes, which requires a much longer collection time. Table III summarises some of the properties of FMN and FAD determined by FCS.

Photobleaching has been noticed in the experiments with either FMN or FAD. In Figs 4a and 4b an example of the number of fluorescence photons emitted by FAD (FMN) during 100 s of constant illumination with 2 mW of

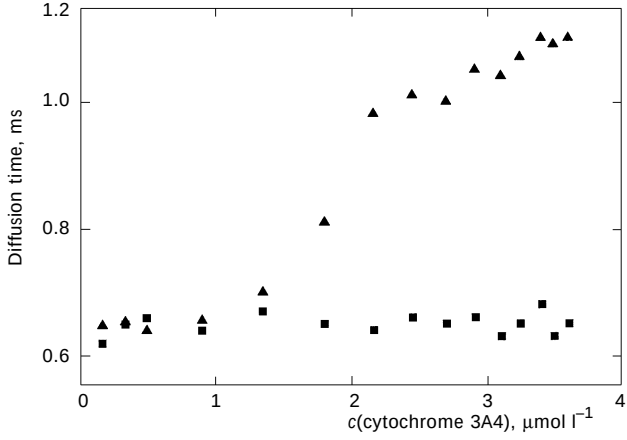


FIG. 2

Dependence of the diffusion time τ_2 (assigned to the coumarin 6/CYP3A4 complex) on concentration of CYP3A4 in the titration of cytochrome with coumarin 6 (τ_1 = 31 μs; 5 nmol l^{-1}): ▲ in water, ■ in the presence of 200 mM ethanol

488 nm Ar^+ laser line is shown. The count rate decreases during these experiments with either FAD or FMN and, concomitantly, the amplitude of autocorrelation function increases. This suggests that the number of fluorescing molecules in detection volume decreases because of the photodestruction.

Assuming an exponential decay of the count rate, photobleaching times T_p for FAD (357 ± 52 s) and FMN (16 ± 3 s) were calculated. FAD appears to

TABLE III
Parameters from FCS experiments on 100 nM FMN and FAD (excitation by an Ar^+ laser 488 nm)

Sample	Diffusion time μs	Triplet fraction %	Estimated particle number[a]	Expected particle number	Count rate kHz
Rhodamine 6G	46 ± 3	7.5	0.32 ± 0.1	0.4	6.9
FMN	39 ± 4	>50	24.0 ± 2.8	26	44.2
FAD	48 ± 3	35	8.7 ± 0.8	26	19.4

[a] From 10 experiments. The structure parameter $S = 5.9$ was determined from the rhodamine 6G data, the detection volume element $V = 4.31 \cdot 10^{-16}$ l.

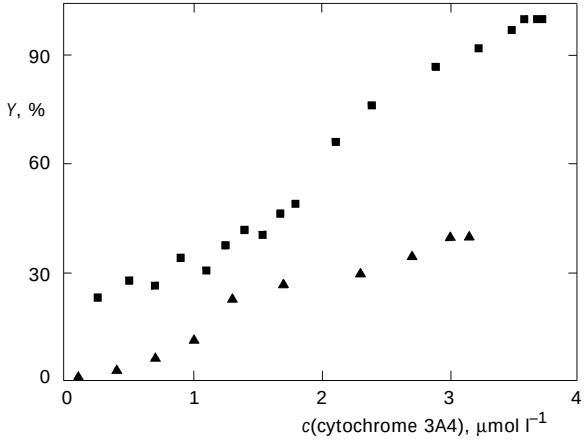


FIG. 3
Fraction Y (%) of the CYP3A4/coumarin 6 complex for titration of CYP3A4 with coumarin 6: ▲ in water, ■ in the presence of 200 mM ethanol

be much less susceptible to photobleaching (Fig. 4) and the fluorescence of FAD is strongly quenched (Table III). The latter observation probably results from dynamic and static interaction of the isoalloxazine ring with the adenine part[42]. The number of FAD molecules in the detection volume (N = 8.7 which is equivalent to 33.4 nmol l^{-1}) is much lower than expected (100 nmol l^{-1}). Apparently, FAD exists for a significant period of time as an intramolecular complex of flavin and adenine[42]. The number of FMN molecules in the detection volume was 24, corresponding to the 93 nM concen-

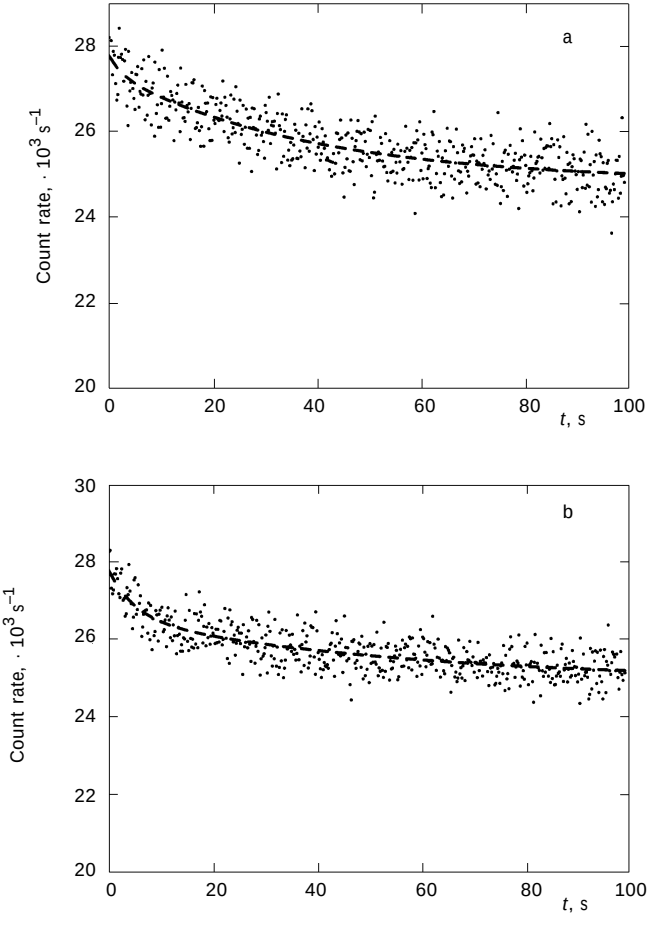


FIG. 4
Number of fluorescence photons emitted by FMN (a) and FAD (b) at concentration of 100 nmol l^{-1} upon 2 mW illumination with the 488 nm Ar^+ laser line. The dash lines are fitted to the function $y = a + b \exp(-t/T_p)$

tration, which is rather close to the analytical concentration of the used solution. This observation is in line with the absence of the adenine moiety in the case of FMN.

Note, that during the preparation of our manuscript a paper by Visser *et al.* was submitted for publication[43]. The authors report FCS results on the photobleaching of FMN and FAD, which appear to be strongly consistent with the data presented in this paragraph.

Interaction of FMN and FAD with NADPH-Cytochrome P450 Oxidoreductase

NADPH-cytochrome P450 oxidoreductase is a flavoprotein, containing coenzymes FMN and FAD. Analysis of the autocorrelation function yielded two components with diffusion times of $\tau_2 = 0.19 \pm 0.03$ ms and $\tau_1 = 0.035 \pm 0.001$ ms. While the latter is apparently due to free diffusing FAD and FMN, the former one is due to diffusion of the NADPH-cytochrome P450 oxidoreductase. Using the expression $D = \omega_1^2/(4\tau_D)$ and values of $\tau_D = 0.19 \pm 0.03$ ms and $\omega_1 = 2.29 \cdot 10^{-7}$ m, a value of $D = 6.92 \cdot 10^{-11}$ m^2 s^{-1} was found, which corresponds to a globular protein of molecular weight 79 000. This finding is in line with the known value of molecular weight of this protein[44] (76 500). We conclude that the shape of the NADPH-cytochrome P450 oxidoreductase is close to a perfect sphere.

The FCS measurement of NADPH-cytochrome P450 oxidoreductase shows significant sensitivity to the power of the laser light (Table IV). In the time period of experiment, the particle number slightly increases from 0.9 at 0.6 mW to 1.1 at 5 mW, which corresponds to concentrations of 5.5 and 6.7 nmol l^{-1}, respectively. At relatively high laser intensities (10 mW), only the diffusion time of free FAD and FMN are observed. The results show that the overall particle number and the contribution of free diffusing molecules (FAD or FMN) to the overall particle number increase with higher laser power. Moreover, even at low laser power (0.6 mW) already a large

TABLE IV
Dependence of the fraction *Y* (%) of the slow diffusing component $\tau_2 = 0.19 \pm 0.03$ ms (component 2 assigned to NADPH-cytochrome P450 oxidoreductase) on the laser power

Laser power, mW	0.6	0.9	1.2	1.5	1.8	2.1	2.4	5
Fraction *Y*	34.5	29.9	18.8	16	13.7	12.6	11.3	5

amount (65.5%) of free diffusing FAD and FMN molecules is detected. This phenomenon can be explained by the principle of photoinduced dissociation of FMN and FAD from the protein[45]. This process apparently occurs on a time scale faster than the time resolution of the experiment, which is determined by the measurement time of 20 s. Similar effects were also observed in surface-enhanced resonance Raman spectroscopic experiments with flavoproteins. Laser illumination during the experiments together with the influence of the adsorbing metal particles on which flavoproteins were bound, were shown to cause the liberation of the flavin prosthetic group from the protein[45].

CONCLUSIONS

Coumarins and resorufins are frequently used as model compounds in metabolic studies of cytochromes P450; hypericin is a specific inhibitor of cytochromes P450 and a constituent of several drugs. Thus the presentation of these compounds as FCS dyes does not only fulfil the need for more suitable probes, but has also some potential physiological use. Using coumarin 6 as a bright label of cytochrome P450 3A4, the tendency of this protein to form aggregates can be estimated by FCS. The studies of the flavoprotein NADPH-cytochrome P450 oxidoreductase as well as of FAD and FMN show that FCS is a useful tool for quantitative characterisation of intramolecular formation of non-fluorescent adducts, photobleaching, and photodissociation.

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