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Safranine O as a Fluorescent Probe for Mitochondrial Membrane Potential Studied on the Single Particle Level and in Suspension

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Abstract—The permeant cationic dye safranine O is often used to measure mitochondrial membrane potential due to the dependence of both its absorption and fluorescence on mitochondrial energization, which causes its oligomerization inside mitochondria. In the present study we have used fluorescent correlation spectroscopy (FCS) to record the fluorescence changes on a micro level, i.e. under conditions permitting resolution of contributions from single particles (molecules of the dye and stained mitochondria). We have shown that the decrease in fluorescence signal from a suspension of energized mitochondria stained with a high safranine concentration (10 μM) is explained by the decrease in dye concentration in the medium in parallel with the accumulation of the dye inside the mitochondria, which results in fluorescence quenching. With 1 μM safranine O, the fluorescence rise after energization is caused by the accumulation of the dye up to a level not sufficient for full fluorescence quenching and also by the higher intensity of mitochondrial fluorescence on immersion of the dye in the hydrophobic milieu. Besides the estimation of the inner mitochondrial membrane potential, this approach also assesses the concentration of fluorescent particles. The non-monotonic dependence of the FCS parameter $1/G(\tau\rightarrow 0)$ on the concentration of mitochondrial protein suggests heterogeneity of the system with respect to fluorescence of particles. An important advantage of the described method is its high sensitivity, which allows measurements with low concentrations and quantities of mitochondrial protein in samples (less than 10 μg).

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Key words: mitochondria, safranine O, potential, fluorescence, FCS, nanoparticles, heterogeneity, suspension

In accordance with the Mitchell's chemiosmotic theory, under conditions of respiratory substrate oxidation mitochondria can generate an electrochemical proton gradient creating a negative potential in the internal compartment of mitochondria, which is a source of energy for ATP synthesis [1]. Hydrophobic permeant cations are used for the registration of mitochondrial potential by different methods, e.g. using ion-selective electrodes, radiolabeled ions, and fluorescent potential-dependent dyes [2]. The latter have been extensively applied in cell biology because they allow the registration of mitochondrial potential in intact cells [3, 4]. A number of potential-dependent fluorescent dyes of different chemical structure have been proposed for the measurement of the membrane potential of isolated mitochondria [5, 6].

Safranine O is a hydrophobic cation that accumulates in mitochondria in response to negative potential generation in the energized state with a concomitant change in absorption and fluorescence spectra [7, 8]. The potential of isolated mitochondria is measured using high concentrations of safranine O (about 10 μM); when an oxidation substrate is added to such a system, the fluorescence intensity drops in response to energization, which is probably due to accumulation of the dye in the mitochondrial matrix in to self-quenching concentrations [7-10].

However, for a suspension of liposomes at a lower safranine concentration (1 μM), it has been shown that generation of the potential results in an increase in fluorescence. This fact has been explained by a model that presumes the mass outflow of the dye molecules into the hydrophobic milieu of the membrane in response to potential generation, which results in the increase in fluorescence intensity [11]. Thus, it can be concluded that the mechanism of fluorescent response of safranine O in

Abbreviations: DNP, 2,4-dinitrophenol; FCS, fluorescence correlation spectroscopy.

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model and native membrane systems is complex and needs further study.

In this study we used fluorescent correlation spectroscopy (FCS) for registration of the fluorescence signal of safranine O in the presence of single mitochondrial particles in a suspension. The FCS method has been widely used in chemistry and biology to study the dynamic state of labeled proteins [12]. The experimental device is a microfluorimeter that collects fluorescence from a small volume of a cell, i.e. 10^{-15} liter [13, 14], which is comparable with the volume of a single mitochondrion. Our data contribute significantly to understanding the nature of the complex dependence of fluorescence of a suspension of mitochondria stained with safranine O on their energization.

MATERIALS AND METHODS

The following reagents were used in this work: safranine O, rotenone, 2,4-dinitrophenol, Mops, EGTA, bovine serum albumin (BSA), bicinchoninic acid, and copper (II) sulfate (Sigma, USA); fluorescent spheres of 0.5 μm size from Molecular Probes (Invitrogen, USA; concentration of $3.65 \cdot 10^{11}$ spheres per ml); sucrose and succinate (ICN, USA); rhodamine 6G (Fluka, USA).

Rat liver mitochondria were isolated by differential centrifugation using a standard procedure. The isolation medium (pH 7.4) contained 250 mM sucrose, 20 mM Mops, 1 mM EGTA, and 1.2 mg/ml BSA. Measurements were performed in the same solution without BSA. Protein was assayed by a standard procedure using bicinchoninic acid and BSA as a standard [15].

Data on the binding of safranine O with mitochondria in the energized and deenergized states were obtained through the measurement of fluorescence of the dye solution before the addition of mitochondria and fluorescence of the supernatant after centrifugation of mitochondrial particles (Eppendorf 5424), 10 min, 9400*g*. The suspension in the energized state contained the proper concentrations of safranine O (0.1-10 μM), mitochondria (0.4 mg/ml), rotenone (2 μM), and succinate (5 mM). The suspension in the deenergized state contained safranine O (0.1-10 μM), mitochondria (0.4 mg/ml), rotenone (2 μM), and KCN (1 mM). The buffer was the same as for the isolation of mitochondria but in the absence of BSA, pH 7.4.

The fluorescence spectra of the dye and the mitochondrial suspension in the energized and deenergized states were recorded in a Panorama Fluorat-02 spectrofluorimeter (Lumex, Russia), excitation wavelength 520 nm and registration wavelength 580 nm, in a standard 2-ml cuvette. For the determination of fluorescence intensities at different dye concentrations, fluorescence spectra were taken at the excitation and registration wavelengths of 520 nm and within 540-740 nm, correspondingly; the integral intensity of fluorescence was calculated as the area occupied by spectral peaks at the same sensitivity of the instrument.

The previously described instrument for fluorescence correlation spectroscopy was used as a microfluorimeter [16]. The dye was excited by a Nd:YAG solid-sate laser, 532 nm, coupled with an Olympus IMT-2 inverted epifluorescence microscope (USA), water-immersion objective 40×, 1.2 NA (Carl Zeiss, Germany). The fluorescence signal was passed through a respective dichroic beam splitter and projected on the 50-μm core of a light guide connected with an avalanche photodiode (SPCM-AQR-13-FC; PerkinElmer Optoelectronics, Canada). The signal was transduced by an interface card (Flex02-01D/C; Correlator.com, USA). For correct operation of the instrument, the position of the light guide relative to the optic axis was adjusted before the experiment. The autocorrelation function for 10 nM rhodamine 6G solution was measured; at optimum, it was characterized by the time of three-dimensional diffusion of about τ_D = 180 μsec. This value makes it possible to describe the meaning of the radius (in the horizontal plane) of confocal ellipsoid as $\omega = \sqrt{4 \cdot D_\tau \cdot \tau_D}$ = 0.42 μm (taking the coefficient of rhodamine 6G diffusion as $D_\tau = 2.5 \cdot 10^{-6}$ cm^2/sec). The data were recorded during 30 sec. Fluorescence records were taken with the sampling interval of 0.2 msec. The fluorescence of the confocal volume located 50 μm above a thin glass coated with 60 μl of mitochondrial suspension was registered.

RESULTS

The dye safranine O is often used for the measurement of the potential of isolated mitochondria due to the dependence of its absorption and fluorescence on energization of the mitochondria. Figure 1 shows a typical record of fluorescence fluctuations in a microvolume expressed as a frequency signal (the number of photons per time unit). The recording board also makes it possible to calculate the autocorrelation function of a record and the mean value of fluorescence from the total confocal volume. The fluorescence signal value depends on the intensity of laser emission, sensitivity of detector, filters used, and the light emittance of fluorophore molecules. For constant performance characteristics of the instrument, the amplitude of fluorescence fluctuations depends primarily on the number of fluorescing molecules per particle. So, for the dye in solution the instrument registers the signal from single dye molecules, and the amplitude of fluctuations is low (Fig. 1a, curve *1*). After the addition of mitochondria in the energized state to the solution of safranine O, the amplitude of fluorescence fluctuations increases due to accumulation of the dye molecules in separate particles, which is recorded as

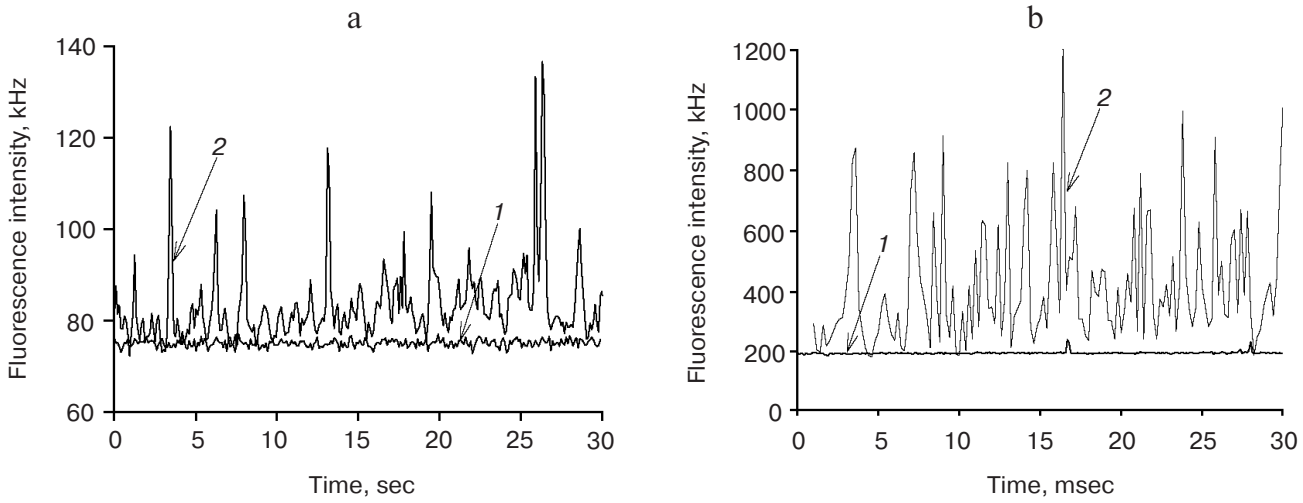

Fig. 1. Record of fluorescence intensity of safranine O solution before (*1*) and after (*2*) the addition of mitochondria without stirring (a) and with stirring (b) recorded by FCS. Concentrations: safranine O, 1 μM; rotenone, 1 μM; succinate, 5 mM; mitochondrial protein, 0.01 mg/ml (a), 0.17 mg/ml (b).

peaks that correspond to the mitochondrial particles carrying a fluorescent label (Fig. 1a, curve *2*).

In the measurement period of 30 sec (Fig. 1a, curve *2*), several events were recorded as peaks with amplitude higher than the amplitude of signals from the dye molecules in solution, which gives insufficient statistics for quantification of light emittance of single particles. Increasing the recording time when measuring the dye fluorescence in a mitochondrial suspension is undesirable as it can lead to the bleaching of the dye and mitochondrial uncoupling. In our study, we stirred the suspension to increase the number of recorded events. Stirring was provided by a rotating blade (rotary speed 600 rpm) attached to an electric motor fixed above the microscopic stage. Figure 1b (curve *2*) presents a much shorter record of safranine O fluorescence intensity in the suspension of mitochondria in the energized state under stirring. At the duration of 30 msec, the record contains many more high-amplitude peaks as compared with the record without stirring for 30 sec. The difference in the amplitudes of peaks on panels (a) and (b) is due to the different laser emission intensities in these two records.

Figure 1 shows that mitochondrial particles in the energized state give a large spectrum of fluorescence amplitudes. This can be associated either with heterogeneity in energization of mitochondria [17] or with the nonuniform distribution of the excitation light intensity within the confocal volume. As a rule, the distribution of laser emission in the confocal volume under single-photon excitation is described by a Gaussian function with a sharp maximum in the center [13]. As a result, the fluorescence amplitude of a particle can be substantially influenced by the position of the latter in the confocal volume. The maximal recorded fluorescence must be observed when the particle crosses the confocal volume strictly on center. Hence, even particles of the same luminance have a distribution in fluorescence intensity due to different distance of their trajectories from the confocal volume center. Figure 2 shows the diagram of the dependence of fluorescence intensity on time for fluorescent 0.5-μm diameter spheres of the same luminance. The spheres carry a fluorescent label; therefore, the FCS signal record is a sequence of peaks with signal nearly absent in between them. As Fig. 2 shows, the fluorescence peaks even for the equally bright particles have substantially different amplitudes, evidently due to the cause described above.

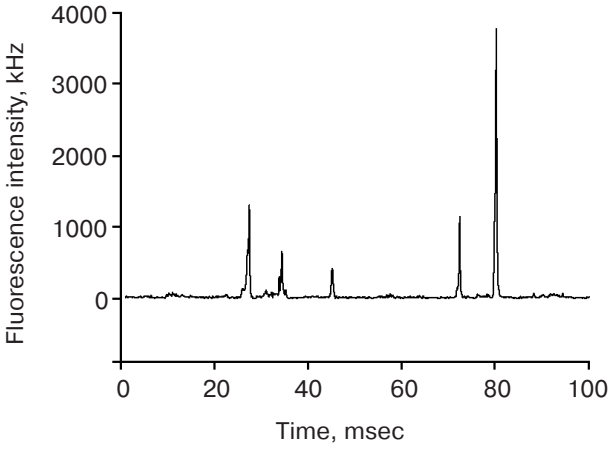

Fig. 2. Record of fluorescence intensity of a suspension of fluorescent spheres of 0.5-μm diameter detected in the FCS instrument.

The standard method of quantitative processing of fluctuating signal $F(t)$ is the calculation of the autocorrelation function, namely:

$$G(\tau) = \frac{\langle \delta F(t)\ \ \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}, \qquad (1)$$

where $\langle F(t) \rangle$ is the mean fluorescence intensity and $\delta F(t) = F(t) - \langle F(t) \rangle$ is the deviation from the mean value.

In a short period of time τ (e.g. for $\tau = 1$ μsec), the probability that a particle will leave the confocal volume is low, the values of fluorescence intensities correlate well with each other, and function $G(\tau)$ has maximal value. With increasing time interval τ, the probability of particle to leave the confocal volume increases and the value of the autocorrelation function tends to zero.

The measurement of the autocorrelation function provides for the quantitative estimation of correlation time τ_d typical of fluorescence signal fluctuations. For the three-dimensional diffusion of particles, function $G(\tau)$ appears as [14]:

$$G(t) = \frac{1}{N} \left(\frac{1}{1 + \frac{t}{\tau_d}} \right) \left(\frac{1}{\sqrt{1 + \frac{w^2{}_0 \cdot t}{z^2{}_0 \cdot \tau_d}}} \right), \qquad (2)$$

where N is the average number of fluorescent particles in the confocal volume; w_0 and z_0 are geometrical characteristics of the confocal volume, and τ_d is the mean time of residence in the confocal volume determined by the size of particles. For the dye (safranine O) bound with mitochondria, τ_d varies from 150 to 300 msec, while for the free dye molecules in solution τ_d is 400 μsec. Figure 3 (curve *1*) shows the autocorrelation function corresponding to the record presented by curve *2* in Fig. 1a.

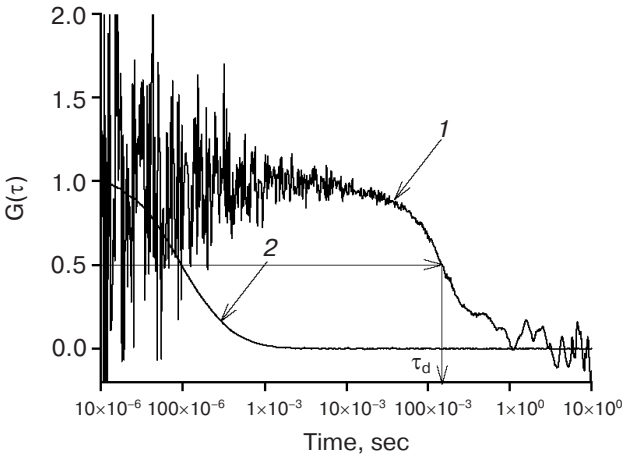

Fig. 3. Normalized autocorrelation function $G(\tau)$ for safranine O (1 μM) in a suspension of mitochondria in the energized state. τ_d is the time corresponding to the half-maximal value for function $G(\tau)$. Curves: *1*) without stirring (τ_d = 160 msec); *2*) with stirring (τ_d = 60 μsec).

The typical time τ_d must significantly depend on whether the measurements are performed with or without stirring of the solution. Figure 3 (curve *2*) presents the autocorrelation function calculated for curve *2* in Fig. 1b (fluorescence of safranine O in the mitochondrial suspension under stirring). In this case, the value τ_d of 60 μsec is determined by the passage of a particle through the confocal volume under the influence of stirring rather than by free diffusion. The value reciprocal to the value of unnormalized autocorrelation function in the limit of small times is proportional to the number of particles in the confocal volume. It is significant that this parameter does not depend on sample stirring [18].

Knowing value $N = (1/G(\tau \to 0))$, one can try to determine the number of mitochondrial particles per mg protein. For this purpose, it is necessary to assess the value of the confocal volume. However, it seems more reliable to measure the dependence of parameter $1/G(\tau \to 0)$ on the concentration of fluorescent spheres. Fluorescent latex particles 0.5 μm in diameter (the size of mitochondria measured by the method of dynamic light scattering in a Zeta-sizer (Malvern Instruments, GB) was 0.45 μm) were taken for this purpose. Figure 4a shows the calibration curve for parameter $1/G(\tau \to 0)$ on the concentration of latex particles (spheres/ml). It can be seen that this parameter increases almost linearly with increasing concentration. A similar dependence was obtained when working with non-fluorescent latex particles in the presence of tetramethyl rhodamine, which is well bound to these particles (data not presented).

Figure 4b shows the similar dependence for a suspension of mitochondria in the energized state at different protein concentrations with the safranine O concentration of 1 μM. In contrast to the fluorescent spheres, the parameter $1/G(\tau \to 0)$ non-monotonically depended on the mitochondrial protein concentration, and its value was minimal at a concentration of about 0.3 mg/ml (Fig. 4b). This result indicates that the suspension of mitochondrial particles in the presence of the dye is a system essentially heterogeneous in luminance, which cannot be reduced to a simple model of a certain number of particles with a certain averaged luminance. Nevertheless, it is reasonable to assess the recorded number of mitochondrial particles by the order of magnitude in a typical experiment in the range of high protein concentrations, when the number of particles is observed to grow with increasing protein concentration. Such assessment yielded a value of about $2 \cdot 10^{10}$ units/mg, which perfectly corresponds by the order of magnitude to values obtained by electron microscopy [19].

Figure 5a shows the record of fluorescence intensity of safranine O (10 μM) stained mitochondria under non-energized, energized, and deenergized conditions. Figure

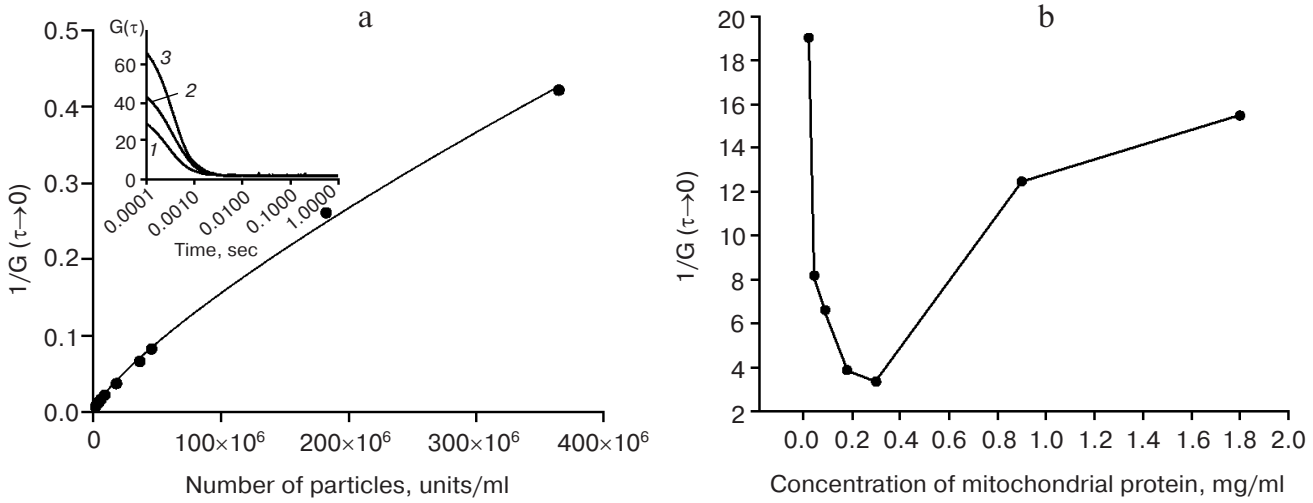

Fig. 4. a) Dependence of the reciprocal value of parameter $G(\tau\rightarrow0)$ on the concentration of fluorescent 0.5-μm diameter spheres. The inset shows the autocorrelation functions measured at the following concentrations of particles per ml: $9.1\cdot10^6$ (*1*); $6.1\cdot10^6$ (*2*); $4.6\cdot10^6$ (*3*). b) Dependence of the reciprocal value of parameter $G(\tau\rightarrow0)$ on protein concentration in a suspension of mitochondria in the energized state. Safranine O, 1 μM; rotenone, 1 μM; succinate, 5 mM.

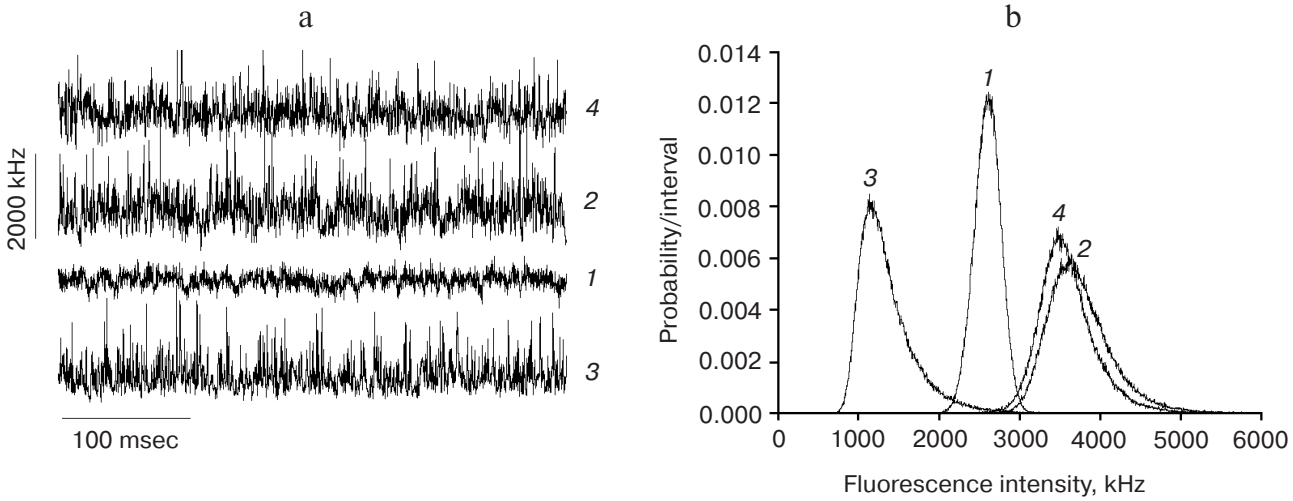

Fig. 5. a) Records of fluorescence intensity of 10 μM safranine O in a mitochondrial suspension detected in the FCS instrument. b) Respective histograms of fluorescence intensity distribution. Curve *1* in panel (a) (histogram *1* on panel (b)): 10 μM of safranine O in the buffer. Curve *2* in panel (a) (histogram *2* on panel (b)): addition of 0.39 mg/ml mitochondria, rotenone 1 μM. Curve *3* in panel (a) (histogram *3* on panel (b)): addition of succinate, 5 mM. Curve *4* in panel (a) (histogram *4* in panel (b)): addition of DNP, 100 μM.

5b presents histograms of distribution of fluorescence intensity for the four records shown in Fig. 5a. Curve *1* corresponds to the record of fluorescence of the dye in solution, showing the fluctuations with the amplitudes corresponding to fluorescence fluctuations of the dye molecules. This follows from analysis of the corresponding autocorrelation function. On addition of mitochondria and rotenone (non-energized state, curve *2*), the fluorescence record curve shows peaks of high amplitude (the right arm on the histogram of distribution of intensities), which correspond to mitochondrial particles probably stained due to unspecific binding of safranine O. The addition of succinate results in the generation of potential (energized state) and in a drop in the total level of fluorescence (curve *3*); at the same time, the amplitudes of the recorded peaks do not change noticeably. The addition of the uncoupler 2,4-dinitrophenol (DNP) to the concentration of 100 μM causes deenergization of mitochondria, release of the dye, and return of the total level of fluorescence to the value observed in the non-energized state, with maintenance of peaks of the same amplitude.

Fluorescent correlation spectroscopy allows estimation of fluorescence intensity both on the macro level, i.e. the average level of fluorescence of the entire confocal volume, and on the micro level, i.e. on the analysis of specific peaks. In the presence of 10 μM safranine O, the fluorescence intensity decreases on energization from 3.6 MHz (in the absence of oxidation substrates) to 1.3 MHz (after succinate addition) and then returns to 3.5 MHz after the addition of the uncoupler DNP. These results are in good agreement with data obtained in a conventional fluorimeter (Fig. 6), where the addition of succinate to mitochondria also results in decrease in safranine O fluorescence intensity, while the dissociation of mitochondria results in the recovery of fluorescence level.

The amplitudes of the peaks were analyzed by calculating the total intensity of all fluorescence peaks in the energized and deenergized states (Fig. 7) as a sum of fluorescence intensities in each point; while the value of fluorescence from the dye in solution was deduced according to Eq. (3):

$$S = \sum \left(F(t) - F_0 \right) , \qquad (3)$$

where $F(t)$ is fluorescence intensity in the moment of time t and F_0 is the intensity of fluorescence of free dye molecules corresponding to the maximum on the histogram of intensity distribution. The processing of records in Fig. 5 showed that the total fluorescence intensity of the mitochondrial suspension was $S = 2.3 \cdot 10^7$ units in the energized state and $S = 3.2 \cdot 10^7$ units in the deenergized state. Consequently, the higher luminance of single mitochondria, i.e. the higher amplitude of fluorescence peaks, was not observed for 10 μM safranine O in the energized state as compared with the deenergized state.

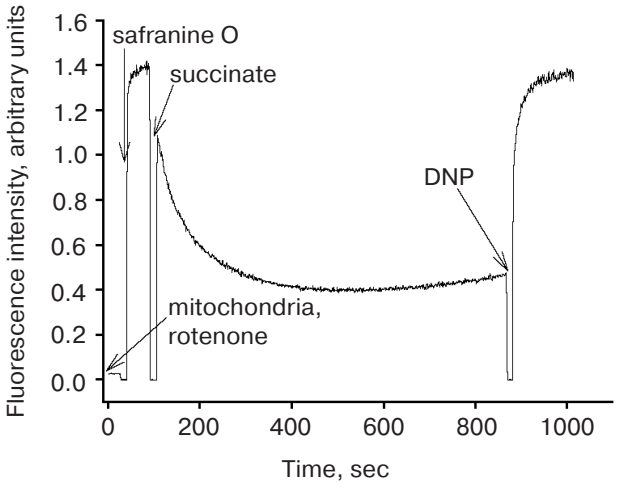

Fig. 6. Record of the fluorescence of 10 μM safranine O in a mitochondrial suspension (0.4 mg/ml) in a conventional fluorimeter. Additions: rotenone, 1 μM; succinate, 5 mM; DNP, 100 μM.

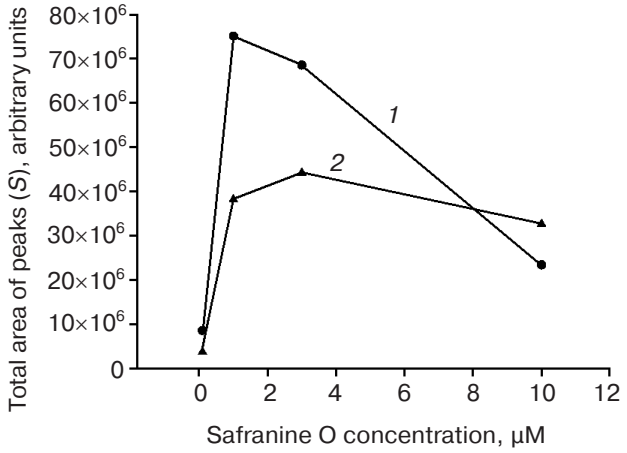

Fig. 7. Dependence of total area of fluorescence peaks (S) recorded in the FCS instrument on safranine O concentration in the mitochondrial suspension in energized (*1*) and deenergized (*2*) states. Conditions: mitochondria, 0.51 mg/ml; rotenone, 1 μM; succinate, 5 mM. Deenergized state was reached by the addition of 100 μM of DNP.

Figure 7 shows the data of such measurements obtained at different concentrations of the dye in solution. One can see that at low concentrations of safranine O (0.1-3 μM) at the same protein concentration deenergization results in a decrease in the total fluorescence intensity as compared with the energized state, i.e. a decrease in luminance of single particles. This can also be seen from the record of fluorescence intensity of a mitochondrial suspension at safranine O concentration 1 μM (Fig. 8a). In the non-energized state, some of the peaks are evidently due to nonspecific binding (Fig. 8a, curve *1*). The addition of succinate leads to an increase in fluorescence of mitochondrial particles and, as a result, an increase in the amplitude of the peaks (curve *2*); the addition of DNP induces deenergization, release of the dye from all particles, and decrease in the peak amplitudes (curve *3*). The amplitude increase can be seen on the histogram of intensity distribution (Fig. 8b), where the right arm of high intensities appears in the energized state (curve *2*).

The analysis of dependence of the fluorescence of mitochondrial particles on their energization at different concentrations of safranine O requires information on the concentration dependence of fluorescence of this dye in aqueous solution. Since we failed to find such data in the literature, we performed these measurements in the present study (Fig. 9). The insert in Fig. 9 shows the concentration dependence of the area under the spectrum of safranine O fluorescence (540 to 740 nm), which has a maximum at the safranine O concentration of about 20 μM. At a concentration of the dye more than 100 μM there is a noticeable shift of the maximum to longer wavelengths.

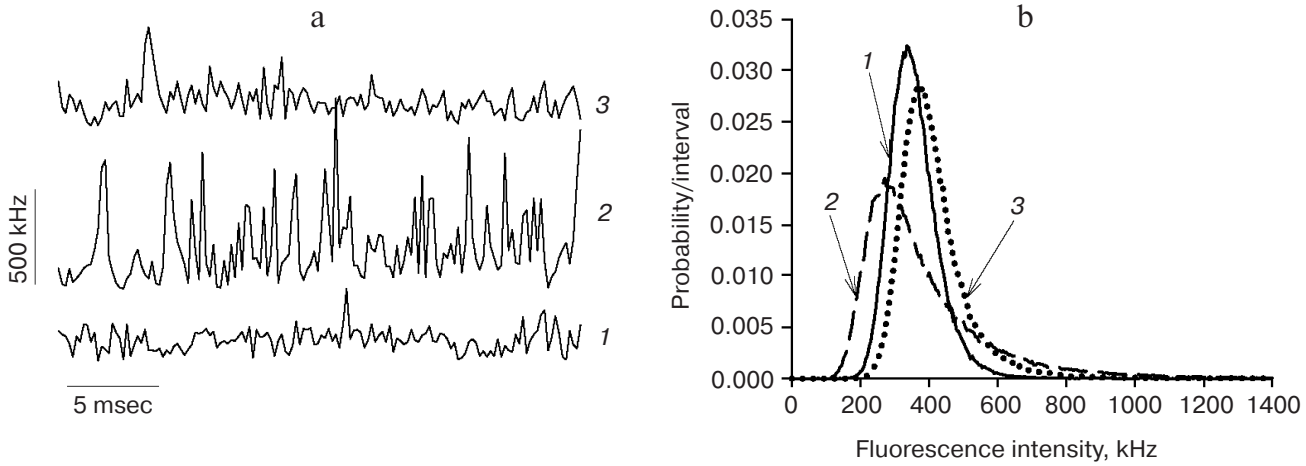

Fig. 8. a) Record of fluorescence intensity of 1 μM safranine O in a mitochondrial suspension in the FCS instrument: *1*) mitochondria, 0.17 mg/ml; rotenone, 1 μM; *2*) addition of 5 mM succinate; *3*) addition of 100 μM DNP. b) Respective histograms of fluorescence intensity distribution.

DISCUSSION

In this study we have used a new approach for the assessment of energization of mitochondria by recording the fluorescence of safranine O from single particles in suspensions of isolated mitochondria. Standard fluorometric measurements with high safranine concentration (10 μM) were conventionally used to measure the membrane potential in mitochondrial suspension on the macro level [7-10]; in this case, fluorescence intensity decreases in response to generation of the potential on energization of particles (Fig. 6). As shown in Fig. 5a, on the micro level, on measurement of fluorescence from single particles (molecules of the dye and stained mitochondria), at high concentration of safranine O (10 μM), energization also results in a decrease in the total level of fluorescence; however, there is no change in the amplitude of peaks and, consequently, no change in the fluorescence of single mitochondrial particles. Based on the microfluorimeter data, it can be concluded that the decrease in the signal on the macro level is associated with the decrease in dye concentration in the external solution of the mitochondria.

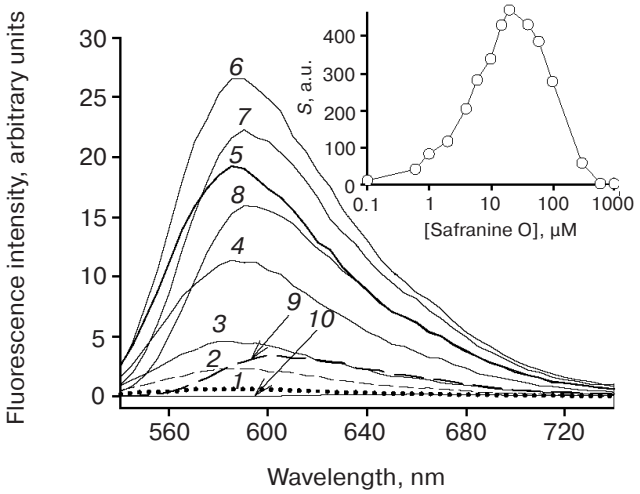

Fig. 9. Fluorescence spectra of safranine O in aqueous buffer solution. Curves: *1-10*) 0.1, 0.6, 1, 4, 10, 20, 60, 100, 300, and 1000 μM safranine O, respectively. Excitation wavelength, 520 nm. Inset: dependence of area under the spectrum on dye concentration.

To gain insight into the processes that occur on energization of mitochondria in the presence of safranine O, one should examine the model taking into consideration concentration quenching of fluorescence in association with oligomerization of the dye [5, 20]. The model envisages the presence of the dye both in the external and internal volume of mitochondria and, in the bound state, on the external and internal sides of the inner mitochondrial membrane. The aqueous solution was suggested to contain a fluorescent monomeric form and a non-fluorescent dimeric form of the dye. This model qualitatively explains the observed decrease in fluorescence of a number of dyes in response to energization of mitochondria, and in some cases it can even quantitatively estimate a number of constants. It can also be used for calculation of the dependence of fluorescence on the membrane potential [5].

It should be noted that the dependence of fluorescence of safranine O in solution on concentration (Fig. 9) cannot be explained within the limits of the assumption of the existence of only fluorescent and non-fluorescent forms of the dye. Such model predicts only the deviation of concentration dependence of the signal of safranine fluorescence from linear dependence and cannot explain the decrease in the signal at high concentrations observed

experimentally (Fig. 9). In order to explain the maximum of the concentration dependence of fluorescence for another dye (carboxyfluorescein), it has been assumed that energy transfer from monomeric to dimeric, the non-fluorescent form of the dye, becomes effective at high concentrations [21]. It can be suggested that a similar process occurs for safranine O as well. It should be noted that just the presence of a maximum in the concentration dependence of fluorescence makes it possible to understand the absence of increase in the observed signal from mitochondrial particles (Fig. 5a).

Thus, our experimental data will be discussed within the framework of a model concerning the presence of the dye in four loci (two in the membrane and two in solution) based on the data on the concentration dependence of fluorescence (Fig. 9). Safranine O, being a positively charged hydrophobic cation, can bind to the inner membrane of mitochondria in the non-energized state as it contains negatively charged lipids. This fact explains the presence of peaks in the absence of energization (Fig. 5a).

The addition of succinate to such system induces the generation of membrane potential, which is negative on the inner side of the membrane, resulting in electrophoretic accumulation of dye molecules inside the mitochondria and its transfer from the membrane to the mitochondrial matrix. Depending on the external dye concentration, the internal concentration can reach values causing its self-quenching (i.e. over 20 μM). As a result of addition of the uncoupler, the potential drops and the dye molecules are released again to the solution.

For the quantitative determination of distribution of the dye inside and outside the mitochondria, direct experiments were performed measuring safranine O binding (see "Materials and Methods") in the energized and deenergized state (see table). The table shows that after incubation with 10 μM safranine O in the presence of succinate only 14.4% of the initial dye concentration was retained in the solution. For assessment of the dye concentration inside the mitochondria, assume the membrane potential to be −180 mV [22-24]. Then, using the Nernst equation and taking into account 1.4 μM safranine O retained in the external solution (table), the internal concentration can be estimated as 1.7 mM. According to Fig. 9, the dye at such concentration displays practically no fluorescence, and the fluorescence of mitochondrial particles can be associated only with the fraction of the dye bound to the membrane. Thus, self-quenching of the dye inside the mitochondrial particles in response to energization results in a decrease in the total level of fluorescence both on the macro and micro levels due to decrease in the dye concentration in the external solution. Independence of the amplitude of peaks on energization can be due to nonspecific binding of the dye to the membrane at such a high concentration, which causes dye saturation.

Now let us analyze in a similar way the case of intermediate concentration of safranine O in solution (1 μM). The data on the binding (table) show that in this case 27.4% of the dye is retained in the solution, i.e. its concentration is 0.27 μM. Assuming that the potential value on the membrane is −180 mV, we obtain the internal dye concentration of 340 μM. The comparison of fluorescence intensities from the data of the inset in Fig. 9 leads to a conclusion that the fluorescence intensity at 340 μM of safranine is higher than at 0.3 μM (the increment is twofold). This fact can explain the increase in the amplitude of peaks in record *2* of Fig. 8a. At the same time, the total signal increases insignificantly (from 350 kHz in the non-energized state to 390 kHz after succinate addition), although the external dye concentration drops substantially (from 0.67 to 0.27 μM; table). This is probably associated with the fact that energization under these conditions results in a certain increase in the fraction of the dye bound to the membrane, which has a high quantum yield of fluorescence in the hydrophobic environment. In accordance with these data, measurement on the macro level also shows a certain increase in the signal (by 25%, taking the level in the energized state as 100%). It should be noted that the observed increase in peak amplitudes (twofold, Fig. 7) is in good agreement with the expected increase in fluorescence (twofold), which has been assessed by the concentration dependence of fluorescence for safranine O in solution (Fig. 9). This occurs in spite of the fact that in the mitochondrial membrane there is a certain portion of nonspecifically bound dye, the fluorescence of which changes much less on energization, which can reduce the differences in the amplitudes

Binding of safranine O with mitochondria

Concentration of safranine O in solution, μM	Fraction of safranine O accumulated in mitochondria due to energization, % of initial concentration in solution	Fraction of safranine O nonspecifically bound to mitochondria in the deenergized state, % of initial concentration in solution
0.1	45.0	27.2
1	72.6	32.6
10	85.6	5.0

of peaks in the energized and deenergized states. The presence of nonspecifically bound dye (along with limitation by instrument sensitivity) prevents great differences in the amplitudes of peaks on energization through decrease in safranine concentration in solution. On application of tetramethyl rhodamine (TMRE) dye with much less expressed self-quenching, this difference attains much greater values [16].

As mentioned above, with safranine O used as a potential-dependent dye in a system with liposomes, generation of the potential (negative inside) results in a significant increase in the total fluorescence signal [11]. In these experiments, the safranine concentration was 1 μM. In view of these data, this result might be associated with the presence of a much smaller internal volume of liposomes as compared with mitochondria due to their much smaller size. The higher surface-to-volume ratio must enhance the role of membrane-bound fraction of the dye, which should induce the increase in fluorescence signal on inside accumulation of the dye.

The conditions when the self-quenching of safranine O is absent make it possible to analyze mitochondrial particles in different states as single events. It opens up new possibilities for population analysis in the suspension of isolated mitochondria on the micro level. The previous attempts for such analysis using a flow cytofluorimeter have shown that the prerequisite condition is a proper ratio of the dye concentrations to mitochondrial protein [25]. The present study demonstrates an apparent heterogeneity in energization of mitochondria, which varies under different conditions. The approach described in this study is in many aspects similar to the principle of the fluorimeter, but the used FCS instrument has a more focused fluorescence excitation beam, allowing a significant increase in measurement sensitivity. The consequence is nonuniform light intensity through the confocal volume. One can hope that further work will make it possible to overcome this drawback, and population analysis of mitochondrial energization will become a routine method of mitochondrial studies.

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