Pteridines
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Characterization of Pteridines: a New Approach by Fluorescence Correlation Spectroscopy and Analysis of Assay Sensitivity

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Abstract

In the present investigation, fluorescence correlation spectroscopy (FCS) was used to measure the molecular motion of the pteridine derivative neopterin. However, technical limitations in the present optical setup precluded the identification of single neopterin molecules. FCS measurements with a fluorophore were also carried out for comparison. Exemplified by rhodamine green, we have introduced a concept that allows the detection, identification and analysis of assays in solution at the single-molecule level in terms of bulk concentration. This concept is based on FCS and Poisson distribution analysis of assay sensitivity. The molecules had not to be quantified in a more concentrated form, or in flow and trapping experiments. The study demonstrated an ultrasensitive, reliable, rapid and direct tool for analytics and diagnostics in solution. We discuss a possible application of our new concept in activation control of cell-mediated immunity via neopterin determination.

Introduction

Pteridines are a fundamental group of natural heterocyclic compounds, which are structurally related by the pyrazinopyrimidine moiety (1). Biochemically active pteridine compounds include vitamins, folic acid, and riboflavin, and cofactors for redox reactions in humans, e.g., 5,6,7,8-tetrahydrobiopterin (2). In human macrophages/monocytes interferon-γ activates GTP-cyclohydrolase I, and increased amounts of neopterin are produced from GTP via dihydroneopterin triphosphate (3). Therefore, neopterin is a measure of the activation level of cell-mediated immunity. In humans, the monitoring of neopterin concentrations in body fluids is especially helpful for follow-up of immunological complications in patients with HIV infection, in chronic infections, to detect immunological complications in allograft recipients and therapy control in autoimmune diseases (4, 5). In very early events of the disease, minute amounts of neopterin precede the clinical manifestation and correlate with the activity of the disease (e.g. in HIV infection). In this contribution, we tried to measure the molecular motion of neopterin by fluorescence correlation spectroscopy (FCS). We also performed FCS measurements with the fluorophore rhodamine green for comparison. We have introduced fluorescence correlation spectroscopy as an ultrasensitive, rapid and reliable methodology for monitoring assay sensitivity on the single-molecule level (6, 7). We describe our new concept for the detection, identification and analysis of single molecules in solution.

Materials and Methods

Reagents

Neopterin (molecular mass 253.2 g/mol) was from Schircks Laboratories (Jona, Switzerland), and we used a freshly prepared stock solution of 10.5 mg neopterin in 100 ml aqua bidestillata (Mayrhofer, Linz).

The fluorescent dye rhodamine-green™ (Molecular Probes Europe BV, The Netherlands, R-6113) was used throughout the study. The excitation and emission spectra of the fluorophore were recorded. They were compatible with the excitation wavelength of 488 nm and the spectral separation of the fluorescence emission measured between 505 nm and 550 nm. For single molecule dilution, we used bidistilled water (Fresenius, Vienna, Austria).

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All FCS measurements were performed in chambered cover glass (Nalge-Nunc, IL, USA) with sample volumes of 20 µl and less.

Optical setup

The optical setup of the fluorescence correlation spectrometer Confocor 2/LSM510 (Zeiss, Germany) is shown in Fig. 1. An argon-ion laser (458 nm, 488 nm, or 514 nm) and two helium-neon laser (543 nm and 633 nm) are available for the excitation of sample molecules. We carried out the experiments with the 458 nm and 488 nm laser lines. For this reason, we used proper excitation filters, which select the laser lines from the beam of the argon-ion laser, and emission filter (transmission above 475 nm and transmission between 505 nm and 550 nm, respectively). The key element in the optical path is the main (dicroic) beam splitter. It reflects the excitation light and transmits the fluorescence light coming from the sample molecules. The laser light is focused by the microscope objective (C-Apochromat 40x/1.2 W Korr) to a nearly diffraction-limited spot within the sample. The sample is placed on the stage of an inverted microscope (Axiovert 100) by a chambered coverglass (sterile borosilicate, Nalge-Nunc, IL, USA), which is fixed in a holder. The fluorescence emissions are collected by the same objective (numerical aperture 1.2). Pinhole optics images the transmitted fluorescence light on the pinhole (size of 70 nm used). After passing a secondary beam splitter, residual laser excitation light and Raman scattered light are cut off by an additional band-pass filter (emission filter). The fluorescence is focused on an actively quenched avalanche photodiode (photon counting mode, EG&G). A personal computer (PC) operating under Microsoft Windows NT is interfaced to the photon-detecting

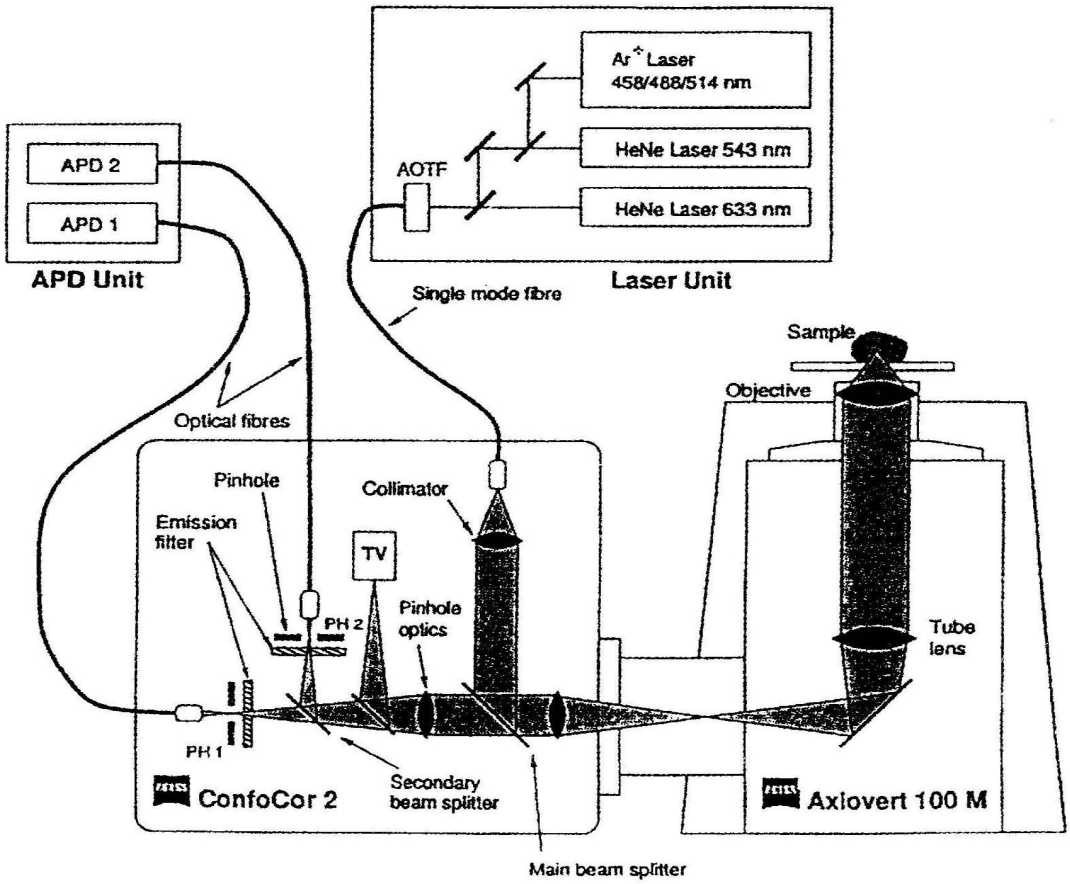

Figure 1. Optical apparatus used. The laser beam (458 nm or 488 nm line) is focused with epi-illumination optics to a small spot in the sample solution. The main (dichroic) beam splitter reflects the excitation light towards the objective and transmits the fluorescencen light originating from the sample. Fluorescence light collected by the objective passes through the main (dichroic) beam splitter. The light filtered by emission filters (see Materials and Methods, Optical setup) is directed to a pinhole that blocks scattered excitation light and fluorescence light from outside the focal spot. Fluctuations in the filtered light are measured by a photodiode

electronics. The software carries out autocorrelations of the photon counts (experimental data points).

Data analysis - FCS data points

The autocorrelation function expresses the average correlation of a concentration fluctuation of a molecule at a certain position and time t with a concentration fluctuation of the same molecule or of any other molecule at a different position and a later time ($t + \tau$) in the volume element V_g (8). The normalized autocorrelation function $G(\tau)$ of fluorescence intensity fluctuations, assuming a random, stationary signal, is (Eqn. 1) where N is the average number of fluorescent molecules observed and tD is the characteristic translational

(Eqn.1)

$$G(\tau) = \frac{1}{N} \cdot \left[\frac{1}{\left(1 + \frac{\tau}{\tau_D}\right) \cdot \sqrt{1 + \left(\frac{\omega_{x,y}}{\omega_z}\right)^2 \cdot \frac{\tau}{\tau_D}}} \right] + 1$$

diffusion time of the fluorescent molecules. wx,y is the radius and wz is the half length of the elliptically-shaped confocal volume element. Deviations between measured (experimental) and calculated (by Eqn. 1) autocorrelations were analyzed with software based on the Marquardt nonlinear least-squares parameterization for calculating the normalized mean square (9). In each analysis the deviations were randomly and close to 1.0.

Poisson analysis of measured G(0) values (6, 7)

The number of fluorescent molecules in solution is always an integer or zero. Let us assume that the solution contains an average number C of fluorescent (e.g., fluorophore) molecules per unit volume V_g (confocal volume of detection). x represents the 'actual' number of fluorescent molecules in the unit volume at an average frequency $\mu = C$. The value x is always a small interger with $x = 1$. Then, the probability $P_{x=1}$ that the confocal volume of detection contains a single fluorophore molecule is given by

$$\ln P_1 = \ln C - C \qquad \text{(Eqn. 2)}$$

For

$$C << e^{-C} \qquad \text{(Eqn. 3)}$$

follows

$$P_1 \cong C \qquad \text{(Eqn. 4)}$$

Thus, we obtain

$$c = \frac{N}{N_A \cdot V_g} = \frac{C}{N_A \cdot V_g} \qquad \text{(Eqn.5)}$$

where c is the average fluorophore concentration of the bulk solution, N_A the Avogadro's number with 6.022 x 10^{23} mol^{-1}, and V_g the confocal volume of detection (unit volume). This treatment enables us to compare the measured value of N with the calculated average frequency C that the volume of detection contains a single fluorophore molecule. N is obtained from the $G(0)$ value. Then, the probability that the unit volume V_g contains two fluorophore molecules ($x = 2$) is

$$\ln P_2 = 2 \ln C - \ln 2 - C \qquad \text{(Eqn. 6)}$$

Results

We applied fluorescence correlation spectroscopy to study molecular diffusion of neopterin. The results are shown in Fig. 2. The concentration correlation analysis yielded a diffusion time for neopterin of 24 µs (Fig. 2A). This diffusion time was well related to the rate of decay of correlation. The faster a species diffuses, the shorter the correlation persists. In contrast, the solvent alone did not reveal any measurable correlation (Fig. 2B). However technical limitations in the present optical setup have precluded the identification of single neopterin molecules. Excitation of neopterin molecules close to the excitation maximum at 353 nm and detection close to 438 nm emission would extend our analysis to the single-molecule domain.

Next, we performed FCS measurements with the fluorophore rhodamine green. During the lifetime of the excited electronic singlet state, the rhodamine green molecules emitted quantum bursts of fluorescent light. The fluorescence process was cyclic as long as the fluorophores were not irreversibly destroyed. The Brownian motion of the fluorophore molecules was observed as fluctuations of the emitted fluorescence intensity within the confocal volume element of detection ("light cavity"). The measurement time was 2 seconds. In Fig. 3 the measured and calculated autocorrelation functions $G(\tau)$ of the fluorophore molecules rhodamine green are presented. The characteristic diffusion time of 28 µs for the fluorophore molecules was short enough to prevent any photobleaching. Under

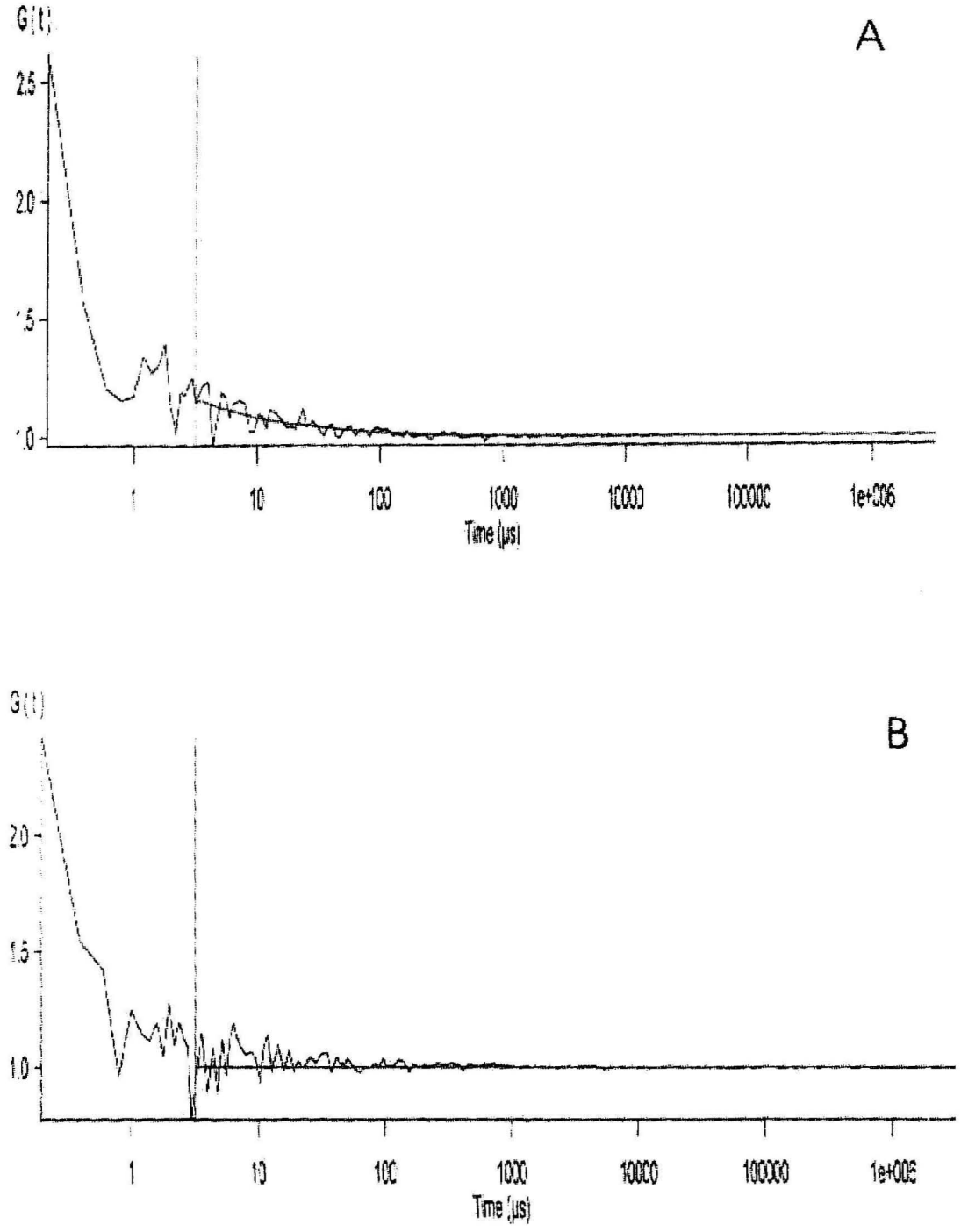

Figure 2. Autocorrelations of the autofluorescence of neopterin measured in a stock solution. The measured curves are depicted in black. The red curves represent the autocorrelation functions of Eqn. 1. We only consider the physical process of three-dimensional diffusion of neopterin through the volume element of detection. A: Stock solution of neopterin. B: Solvent (aqua bidestillata) alone

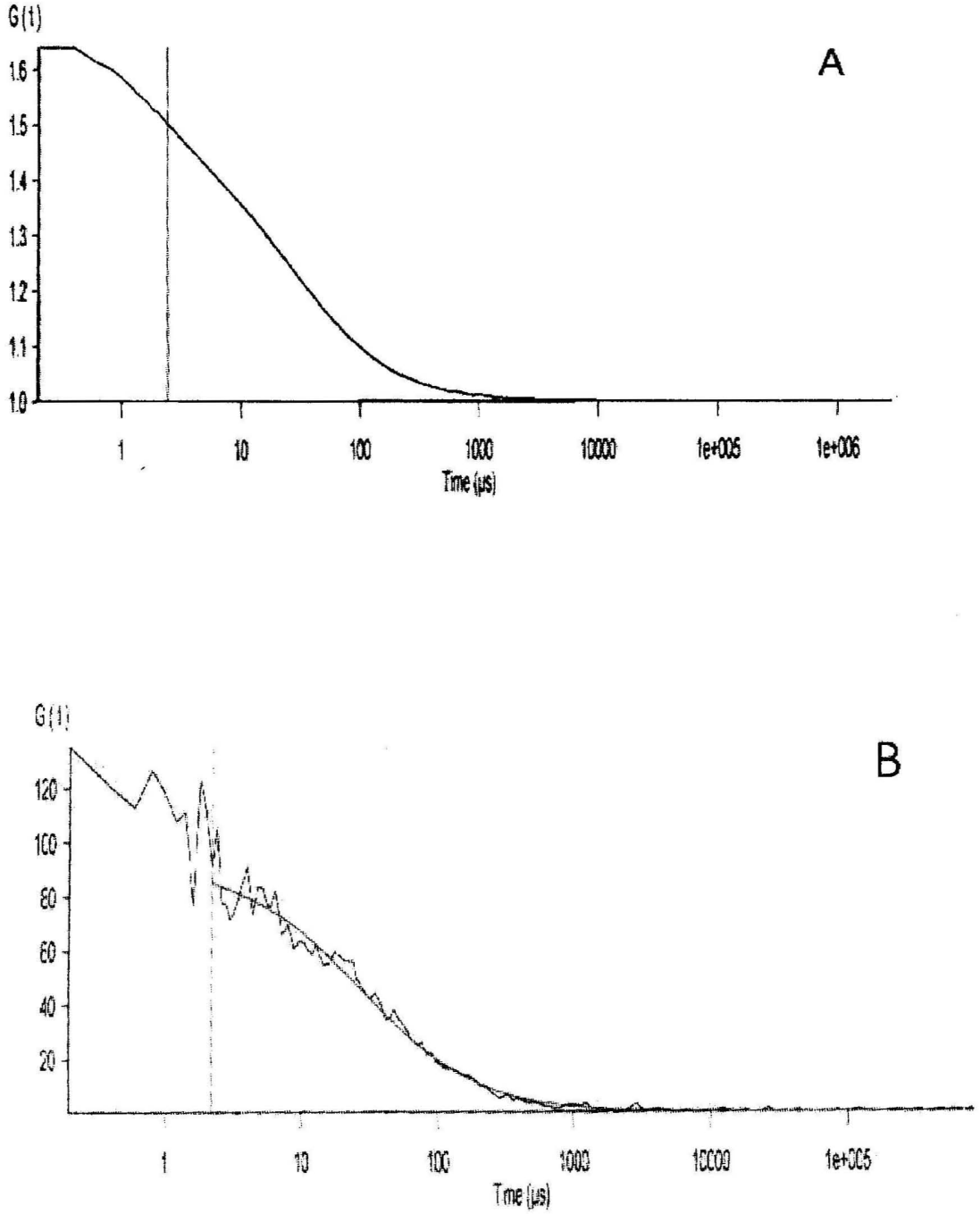

Figure 3. Autocorrelations of the green fluorescence intensity fluctuations measured with sample times 2 s. The measured curves are depicted in black. The red curves represent the autocorrelation functions of Eqn.1. We only consider the physical process of three-dimensional diffusion of the fluorophore rhodmaine green through the volume element of detection. A: Bulk solution of 20.116 nM rhodmaine green. B: Bulk solution of 0.109 nM rhodmaine green.

Table 1. The experimental values of *G(0)* are compared with the average frequency assigned to them to detect a single fluorophore molecule. On this basis, the parameter P_2 is obtained. For explanation, see text.

Mean fluorescent intensity (Hz)	Absolute photon counts per molecule and second	$N_{measured}$*	$C_{calculated}$	P_2
247229	122209	2.023	n.d.**	n.d.
9565	116646	0.082	0.090	1: 270
4896	122400	0.040	0.042	1: 1182
3210	118889	0.027	0.028	1: 2625
2138	118778	0.018	0.018	1. 6289
1413	128455	0.011	0.011	1: 16722

* The concentrations of the bulk solutions were 20.116 nM, 0.815 nM, 0.398 nM, 0.269 nM, 0.180 nM, and 0.109 nM, respectively.
** Not defined.

these conditions, the average photon counts per fluorescent molecule and second were 121230 ± 4172 Hz (mean ± SD, n = 6). Using our optical system, the emitted fluorescence intensity was measured without any background noise. The time course of $G(\tau)$ did not change, however the *G(0)* values increased with decreasing bulk concentrations of the fluorescent species. Because of measuring correlated fluctuations of emitted light quanta rather than average intensities, fluctuations became largest if all fluorescence arises from a single source of emission while intensities decreased. The lowest N value measured without any background noise was 0.0085. Given that the probability P_1 to detect a single fluorescent (florophore) molecule in the confocal volume element is Poisson distributed, and that it depend on the average frequency (number) C of fluorescent (fluorophore) molecules per unit volume V_E (volume element of detection), P_1 is of the form of Eqn. 2. We compared the values of Eqn. 2 for small average numbers C with the experimental values obtained from *G(0)* (Tab. 1). The average frequency C for detecting a single fluorescent molecule in solution was almost equal to the experimental value extracted from *G(0)*, if the experimental value for N was less than 0.5 nM. Thus, the P_2 values in Table 1 indicated that we achieved single molecule sensitivity at fluorophore concentrations less than 0.5 nM. The measured value N of 0.011 meant about 7 x 10^{10} fluorophore molecules per ml bulk solution.

Discussion

Fluorescence correlation spectroscopy (FCS) is performed in a confocal optical arrangement with a well-focused laser beam that defines itself the femtoliter "cavity" (10, 11, 12). This enables us, in principle, to measure fluctuations of the fluorescence intensity with negligible background. In contrast to FCS, large volumes of detection are illuminated in conventional fluorescence spectroscopy. The average fluorescence intensities are measured against a high background noise of scattered light and autofluorescence light of the medium. This results in physical limitations of sensitivity and resolution for quantitative measurements below 1 nM. FCS was developed as an alternative way of measuring the translational (8, 13, 14) and rotational (15) diffusion coefficients and chemical reaction rates of molecules in solution. The detection principle

in FCS is based on the observation of the random Brownian motion of fluorescent molecules. Fluorescent molecules are diffusing through a microscopic laser focus. Fluorescent light is emitted during the transient time. Thus, diffusion of fluorescent particles in and out of the femtoliter "cavity" (volume element of detection) results in fluctuations of the fluorescence intensity, which are recorded.

We report here that FCS allows to measure the autofluorescence of neopterin (see Fig. 2). This implies a novel way for the determination of activation control of cell-mediated immunity. However, we could not observe the molecular motion of neopterin molecules at the level of superior assay sensitivity because of an inadequate excitation laser (458 nm laser line instead of a laser line close to 353 nm) and an inadequate emission band pass filter (long band pass above 475 nm instead of a band pass close to the emission maximum of neopterin at 438 nm). After this first demonstration of FCS measurements with neopterin, it is in principle possible to overcome these limitations.

To study individual molecules we used the fluorophore rhodamine green. Its excitation/emission characteristics are well compatible with our present optical setup. Due to the illumination of a sufficiently small volume (confocal volume element of detection) and the optical separation of the emitted fluorescence from scattered excitation light and other interfering luminescence (see Fig. 1), the fluorescent intensities were not superimposed by background noise in the case of rhodamine green. The count rate per fluorescent molecule proves that the fluctuations in fluorescence intensities arise only from single fluorophore particles. The absolute number N of fluorescent molecules in the volume of detection was obtained from autocorrelation of fluorescence intensity fluctuations. N was taken from the inverse amplitude of $G(0)$. Next, we introduced the Poisson distribution analysis in order to predict the sensitivity of an assay at the level of single molecules by concentration units of the bulk solution (6, 7). For this purpose, we first show that the probability to find a single molecule in the "light cavity" (volume of detection) is Poisson distributed under assay conditions less than 0.5 nM of fluorescent molecules (fluorophore, see Table 1). The random or stochastic behavior was not based on measurement errors. The molecules randomly distribute in solution in terms of bulk concentration. Then, the P_2 parameter is the found criterion to monitor assay sensitivity at the single molecule level in terms of bulk concentration. The Poisson distribution analysis of single molecule detection in terms of bulk concentration is an effective and elegant way to judge assay sensitivity (6, 7).

Acknowledgments

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