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High-Sensitivity Single-Molecule Fluorescence Detection in Theory and Practice

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ABSTRACT

The number of emitted photons that can be obtained from a fluorophore increases with the incident light intensity and the duration of illumination. However, saturation of the absorption transition and photodestruction place natural limits on the ultimate signal-to-noise ratio that can be obtained. Equations have been derived to describe the fluorescence-to-background-noise ratio in the presence of saturating light intensities and photodestruction. The fluorescence lifetime and the photodestruction quantum yield are the key parameters that determine the optimum light intensity and exposure time. To test this theory we have performed single molecule detection of phycoerythrin (PE). The laser power was selected to give a mean time between absorptions approximately equal to the fluorescence decay rate. The transit time was selected to be nearly equal to the photodestruction time of ~600 μs. Under these conditions the photocount distribution function, the photocount autocorrelation function, and the concentration dependence clearly show that we are detecting bursts of fluorescence from individual fluorophores. A hard-wired version of this single-molecule detection system was used to measure the concentration of PE down to 10^{-15} M. This single-molecule counter is three orders-of-magnitude more sensitive than conventional fluorescence detection systems. The approach presented here should be useful in the optimization of fluorescence detected DNA sequencing gels.

BIOGRAPHIES

Richard A. Mathies is Professor of Chemistry at the University of California at Berkeley. He holds M.S. and Ph.D. degrees in Physical Chemistry from Cornell University. Dr. Mathies' research has focused on the use of laser spectroscopy to study the structure and dynamics of light driven biological pigments.

Konan Peck is a postdoctoral fellow at the University of California at Berkeley. He holds a B.S. degree from the National Chung-Hsing University at Taichung, Taiwan and a Ph.D. in Analytical Chemistry from the University of Michigan at Ann Arbor where he worked on the development of ultrasensitive chromatographic detection systems.

Lubert Stryer is Winzer Professor of Cell Biology at the Stanford University Medical School. He holds an M.D. from Harvard University. He is author of Biochemistry and a leader in the development of biomolecular fluorescence spectroscopy.

INTRODUCTION

Fluorescence spectroscopy is an ideal method for quantitating the concentration and location of fluorescent molecules because of its high sensitivity. Fluorescence detection is widely used in immunoassay, flow cytometry, and chromatographic analysis where the detection limits range from 10^3 to 10^6 fluorescent molecules (1, 2) and in automated DNA sequence analysis where the detection limits are 10^6 - 10^7 molecules (3, 4). The development of more sensitive fluorescence detection systems is important because it would permit new applications of this technique in analytical chemistry, biology, and medicine. In particular, increased sensitivity should enable the more rapid analysis of fluorescence detected DNA sequencing gels that is needed in the human genome project (5).

In the quest for enhanced sensitivity, Hirschfeld used evanescent-wave excitation to detect an antibody molecule labeled with 80 fluoresceins adsorbed on a glass slide (6). Using a flowing sample, Dovichi et al. (7) achieved a detection limit of 22,000 rhodamine 6G molecules in a 1 s integration time, and Nguyen et al. (8) extended this limit

to 800 molecules with hydrodynamically-focused flows. Mathies and Stryer (9) pointed out the limits imposed by photodestruction and detected three molecules of B-phycoerythrin (PE) in a probe volume of 10 pL. PE is an attractive fluorophore for enhancing sensitivity because of its high absorption coefficient, near unity fluorescence quantum yield, and large emission Stokes shift (10, 11, 12). Recently, Nguyen et al. observed bursts of fluorescence when a 10^{-12} M solution of PE was flowed through a focused laser beam, and they interpreted these bursts of fluorescence as being due to the passage of individual molecules (13). In this paper, we present a theory for optimizing laser excited fluorescence detection and apply it in detecting single molecules of phycoerythrin at concentrations as low as 10^{-15} M.

THEORY

We consider a solution of fluorophores flowing through a focused laser beam as depicted in Fig. 1. The emission from the illuminated volume consists of bursts of fluorescence from molecules passing through the beam superimposed on a continuous background due to Rayleigh and Raman scattering from the solvent and cell. To detect these fluorescent bursts it is first necessary to make them as intense as possible relative to the background. In the search for the optimal light intensity and transit or illumination time we realized that the previous treatments of photodestruction assumed that the photodestruction rate and the rate of fluorescence are linearly related to the incident light intensity. This is true for weak illumination conditions (9, 14) but will not be true for the high light intensity conditions that occur as one tries to detect the passage of single molecules through a tightly focused laser beam. At high light intensities a significant fraction of the molecules will be pumped to their excited states causing ground state depletion. This will alter the signal-to-noise ratio in the experiment by reducing the fluorescence-to-background ratio. Also, the observed rate of photodestruction will depend on the extent of ground state depletion. These factors can complicate the search for the optimal light intensity and transit time. The optical pumping should be intense but once the transition is saturated increasing the light intensity further will just increase the background. Also, illuminating the molecule for a longer time will generate more fluorescent photons per molecule. However, there is no benefit in looking once the molecule has been

photodestroyed. Our theory for selecting the optimal illumination conditions and transit time based on a measurement of the fluorescence lifetime and the photodestruction rate or quantum yield is outlined below (15).

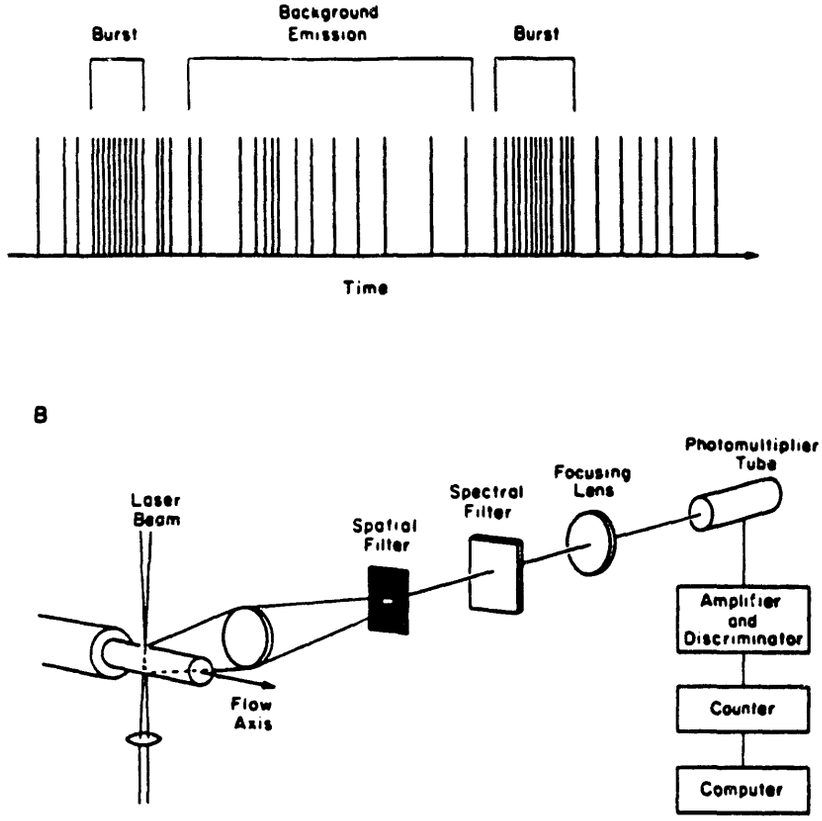

Figure 1. (A) Illustration of the concept of single molecule fluorescence detection. When a fluorescent molecule flows through a laser beam, a burst of photons is generated; single-molecule events can be distinguished from the Poisson distributed background if the fluorescence emission rate is higher than the background emission rate. (B) Experimental apparatus for single molecule fluorescence detection. The 514.5 nm output from an argon ion laser is focused to a 8 μm diameter spot in a capillary tube through which a sample solution is flowed. The resulting fluorescence is collected at 90^0 and passed through spatial and spectral filters to the detection system.

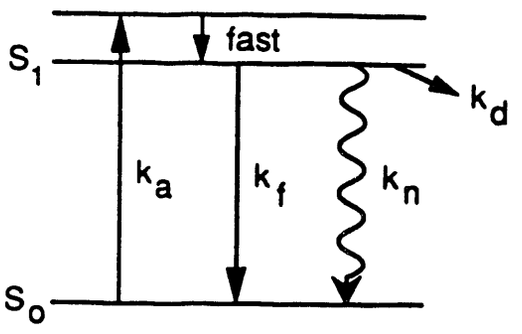

Figure 2. Schematic ground S_o and first excited S_1 singlet state levels. k_a is the rate of absorption, k_f is the natural radiative rate, k_n is the radiationless decay rate, and k_d is the photodestruction rate. It is assumed that relaxation from the optically pumped vibronic levels to the emitting levels is very rapid compared to the pumping rate k_a.

The kinetic scheme that we will use to describe the fluorophores is given in Figure 2. The fluorophore in the ground state S_0 is excited to S_1 by a photon, and the rate of absorption is given by

$$k_a = \sigma_a I = 3.8 \times 10^{-21} \varepsilon I \qquad \{1\}$$

where I is the light intensity (photon cm^{-2} s^{-1}), σ_a is the absorption cross section (cm^2/molecule) and ε is the molar extinction coefficient. S_1 can decay by emitting a photon k_f, through nonradiative decay k_n, or through photochemistry k_d. The observed fluorescence decay rate k_s is determined by the sum of these processes.

$$k_s = k_f + k_n + k_d = 1/\tau_f \qquad \{2\}$$

Here τ_f is the observed fluorescence decay rate. Also, it is useful to define the photodestruction time τ_d as $1/k_d$. The fluorescence quantum yield Q_f and the photodestruction quantum yield Q_d are given by

$$Q_f = k_f/k_s \qquad \{3\}$$

$$Q_d = k_d/k_s \qquad \{4\}$$

We consider a solution flowing with velocity v (cm/s) through an exciting laser beam having a uniform intensity profile in a square of side L. The transit time for the molecule through the beam is $\tau_t = L/v$. Under these conditions the number of photons emitted by the fluorophore during its transit through the beam is given by (15)

$$n_f = (Q_f/Q_d)\ \{1-\exp(-k\tau/(k+1))\} \qquad (5)$$

where we have introduced the dimensionless variables $k = k_a/k_s$ and $\tau = \tau_t/\tau_d$. Meanwhile the scattering background is given by

$$n_b = (Q_f/Q_d)\ k\ \tau\ \alpha \qquad (6)$$

where α is the ratio of the background scattering to fluorescence at very low light levels and short illumination times.

Equations 5 and 6 provided the fundamental relations that permit us to optimize the conditions for single molecule detection. Figure 3 presents a plot of $n_f/\sqrt{n_b}$ which represents the signal-to-background noise ratio when background noise is dominant. The figure makes it clear that the S/N increases rapidly as k and τ are increased but that as k and τ approach unity an approximate plateau is reached. It is not practical to increase the transit time much beyond the photodestruction time because the S/N does not increase very rapidly. Similarly increasing the light intensity beyond that necessary to get the absorption rate equal to the fluorescence decay rate (k =1) is not valuable. These optimum conditions have been used to demonstrate single molecule burst detection of monomers and dimers of PE (16).

EXPERIMENTAL REALIZATION

To explore the detection limits of single molecule counting, PE monomers and dimers from 10^{-15} M to 10^{-12} M were examined with the hard-wired single molecule gating circuit. Because the distribution of the observed count rate is the convolution of background scattering and fluorescence emission, any discriminator setting will necessarily report both real single molecule and false background events. Figure 4 presents the log of the number of single molecule events versus the log of the concentration. The regression lines of these two plots

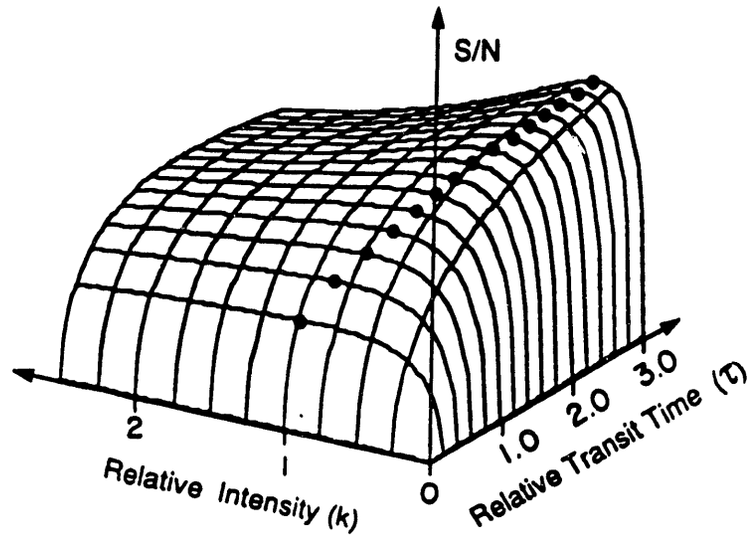

Figure 3. Plot of $F/\sqrt{B}$ in arbitrary units as a function of the relative light intensity k and relative transit time τ. The optimum value at each τ is indicated by the dots.

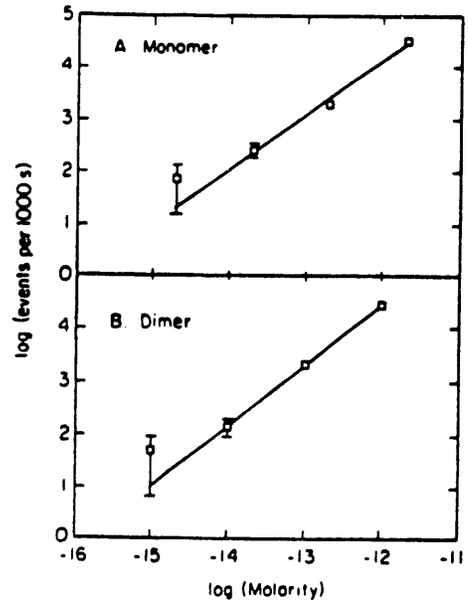

Figure 4. Fluorescence detection limits with single molecule counting. (A) PE monomer with concentrations from 2 fM to 2 pM. (B) PE dimer with concentrations from 1 fM to 1 pM. To reduce the number of false events the discrimination threshold was set so that only about 15% of the actual number of monomer events and 20% of the dimer events were detected.

have a slope fairly close to one, 1.05 for the monomer data and 1.15 for dimer data. The linear concentration dependence strongly supports the idea that we are seeing single-molecule events. The dynamic range is limited by sampling time at low concentrations and by multiple occupancy at high concentrations. At concentrations higher than 10^{-12} M, the mean of the Poisson background will shift up due to the fluorescence from multiple molecules in and around the probe volume. To detect single molecule bursts one must ensure that the probability of observing emission from two molecules simultaneously in the beam is negligible.

In conclusion, we have identified the key parameters for optimizing laser excited fluorescence detection and developed criteria and methods for performing single molecule counting. A hard-wired analog single molecule detection system was used to detect PE monomers and dimers at concentrations as low as 10^{-15} M. This is a 1000-fold improvement over previous sensitivity limits (9). The enhanced sensitivity afforded by single molecule detection should be directly applicable to the detection of fluorescent molecules, fluorescent-labeled peptides or fluorescent DNA fragments in HPLC or capillary electrophoresis (17). The concepts presented here should also be useful in optimizing fluorescence detection systems in DNA sequencing. Toward this end preliminary experiments using 0.25 mm thick polyacrylamide gels with fluorescent labeled DNA sequencing primers have shown a sensitivity of 10^5-10^6 molecules per band. Further improvements in the optical design and gel format to lower this detection limit are in progress.

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