Detection of single fluorescent molecules

E. Brooks Shera [1], Newton K. Seitzinger, Lloyd M. Davis [2], Richard A. Keller and Steven A. Soper
Los Alamos National Laboratory, Los Alamos, NM 87545, USA

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We report the first efficient detection of individual fluorescent molecules in solution. This constitutes a significant advance in ultrasensitive detection that should have a variety of applications in biological and chemical science. The observation technique, which involves excitation with a repetitively pulsed laser and time-gated discrimination of fluorescence photons, permits single molecules to be detected and counted as they transit the laser beam. Our observations are consistent with Monte Carlo modelling of the method. The development is an essential step in the realization of a proposed high-speed method for sequencing DNA.

1. Introduction

In high vacuum, individual atoms and molecules have been observed, studied and trapped for long periods, and an extensive literature describes appropriate techniques and the contributions they have made to scientific understanding in many fields. However, those techniques are not suitable for many biological and chemical applications that could benefit from the observation of single molecules. For example, a recently proposed [1] method of rapid DNA base-sequencing is based on sequential enzymatic degradation of a single DNA strand and identification of the individual cleaved tagged bases in an aqueous environment.

The observation technique we have used involves exciting fluorescent molecules by passing them in solution through a highly focused laser beam and detecting the subsequently emitted photons. The fluorescence lifetimes of the molecules are much shorter than the time the molecule spends in the laser beam, so each molecule is re-excited many times and yields many fluorescent photons. The signature of a passing molecule is the burst of photons that occurs when the molecule passes through the laser beam. A microscope objective and a slit are arranged to image the photons from a small region around the laser beam waist onto a microchannel plate photomultiplier (MCP) operated in single-photon counting mode (fig. 1). The intense excitation light from the laser is blocked from reaching the MCP by a bandpass spectral filter whose transmission band is centered near the peak fluorescence wavelength.

Several previously reported experiments in sensitive detection have used similar methods [2–4], but none have achieved sufficient sensitivity to efficiently detect the fluorescent light from a single molecule. In the most sensitive experiments reported thus far, Peck et al. [4] explored ultra-sensitive detection of the molecular species phycoerythrin, a large compound molecule containing multiple chromophores that is approximately 30 times more fluorescent than single chromophore dyes, such as Rhodamine-6G (R6G) as used in the present experiments. Peck et al. observed a non-random autocorrelation (see eq. (1) below) in their data for runs during which approximately 2000 phycoerythrin monomers passed through their detection volume. They present indirect evidence that they are able to distinguish a fraction (about 15%) of passing phycoerythrin molecules from the background. In the present work we have achieved efficient detection of single chromophore dyes, which should greatly in-

[1] To whom correspondence should be addressed; Physics Division, MS-D434.
[2] Permanent address: Center for Laser Applications, University of Tennessee Space Institute, Tullahoma, TN 37388, USA.

crease the range of potential applications.

To reliably identify the passage of individual molecules the number of detected photons from each molecule must be maximized and the flux of background photons must be low. The background may come from several sources, among them (a) Rayleigh scattered light that is incompletely attenuated by the spectral filter; (b) light from fluorescence of the flow cell, collection optics and the spectral filter; and (c) Raman scattering from the solvent. A carefully designed optical system can largely eliminate the first two background sources but Raman scattering remains.

2. Experimental

We have exploited the temporal differences between Raman scattering (prompt) and the molecular fluorescence signal (delayed) to reduce the Raman background below that in any previously reported experiments. The apparatus we have used is shown in fig. 1. A shortpulse (70 ps), high-repetition rate (82 MHz) pulsed Nd:YAG laser with output wavelength of 532 nm is used as our excitation source. The fast response time of the MCP detector permits us to determine whether a detected photon is coincident or delayed with respect to the laser pulse. Time spectra obtained with this apparatus from two different concentrations of R6G solution are shown in fig. 2. A strong peak at the time of the laser pulse ($t=0$) is due primarily to Raman scattered photons. The prompt peak is followed by an exponential decline in the intensity of the detected light with the lifetime characteristic of R6G. By setting a time-gate window that includes only delayed photons, we can reject the scattering signal and retain the majority of the desired fluorescent signal. For the more dilute solution shown in fig. 2, the ratio of scattered to fluorescence intensities is greater than 100:1. Thus time gating can improve the signal-to-background ratio by more than two orders of magnitude. Also, background from sources that are constant with time (e.g. detector dark noise) or stray fluorescence that decays slowly can be partially rejected by setting a time gate of appropriately short duration.

The time-gated counts from the detector that oc-

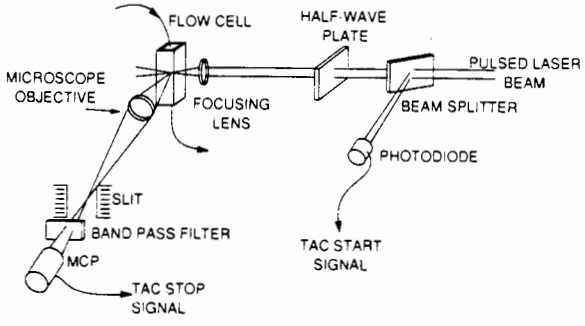

Fig. 1. Schematic drawing of the detection apparatus. Light from a Spectra Physics 3800 mode-locked frequency-doubled Nd:YAG laser was split into two beams. One was focused onto a Hamamatsu S1188 photodiode whose output was shaped by a Tennelec TC454 discriminator that provided the start pulse for a Tennelec 863 time-to-amplitude converter (TAC). The second beam was directed through a neutral density filter to adjust the power level, through a 1/2-wave plate to produce horizontal polarization, and through a 17 mm focal-length lens into a 10×4 mm flow cell, which is fed by a Harvard apparatus 44 syringe pump through a 0.1 micron Millipore filter. Fluorescent light was collected by a 40X 0.65NA objective, spatially filtered by a 0.4 mm vertical slit, spectrally filtered by a 580/40 nm Omega Optics interference filter, and detected by a Hamamatsu R1562U MCP. The MCP signal was amplified by a Hewlett Packard 8447F amplifier, shaped by a Tennelec TC454 discriminator and, with appropriate time delays, fed to the gate and stop inputs of the TAC. The time-gated TAC output was counted in a Joerger S3 CAMAC multiscaler, which was interfaced via a SEC CBD8210 VME interface to a Sun 3/160 workstation.

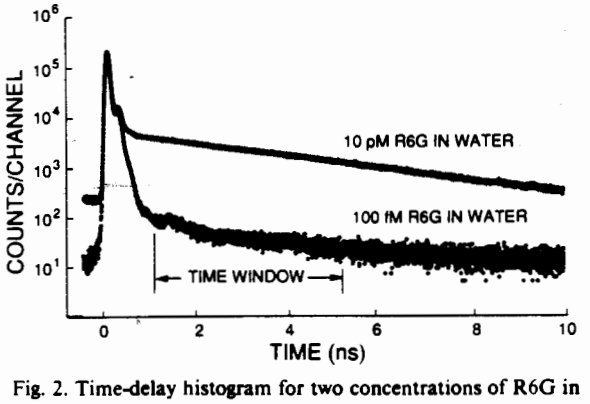

Fig. 2. Time-delay histogram for two concentrations of R6G in water. The curves, which were accumulated for 30 min, show the number of counts recorded as a function of time after the laser pulse. The time-gate window that we have used to reject prompt scattering is shown. The small bump near 1.5 ns is caused by reflected light.

curred in consecutive 4 ms time intervals were accumulated in a multichannel scaler. The data stream from the scaler was analyzed in real-time using a VMEbus-based scientific workstation and was continuously recorded to a disk file for off-line analysis. The 4 ms scaler dwell interval is equal to about 40% of the effective residence time of a molecule in the detection volume. The effective molecular residence time is affected by the velocity of the solvent stream in the flow cell, by molecular diffusion (since molecules may diffuse in and out of the detection volume in addition to being swept through it), and by photobleaching of the dye. In the present experiments we estimated the flow velocity to be 240 μm/s by observation of 0.6 μm diameter fluorescent microspheres, for which diffusion and bleaching are negligible.

A Monte Carlo simulation of the molecular passage process, which used measured values of the flow velocity and quantum yield for photobleaching [5], and a computed diffusion coefficient, indicated an effective residency of 10 ms in our 0.44 pl central detection volume. The latter is defined by the intersection of the laser beam waist (7.5 μm in diameter at 1/e intensity) and the window width of the slit system (10 μm). The effective residency time is about one third of the transit time, largely because, in aqueous solution, most R6G molecules are photobleached after travelling only part of the way through the detection volume.

3. Results and discussion

Investigations of dynamic light scattering [6] have shown that the autocorrelation function is a useful tool for studying the average motions of small particles in solution. For a dataset $d(t)$ consisting of N consecutive values of the number of photons detected in a specified time interval, the digital autocorrelation function is given by

$$A(\tau)=\sum_{t=0}^{N-1} d(t)\,d(t+\tau)\,. \qquad (1)$$

The function $A(\tau)$ is plotted in fig. 3 for data streams obtained from water, with and without the addition of R6G. The peak near $\tau=0$, which increases in prominence for more concentrated solutions and is much diminished when no fluorescent molecules are added to the solvent, is evidence that we are observing brief increases in light intensity due to the passage of R6G molecules. When an oxidant, such as $KMnO_4$ (5 μM), is added to the solvent to degrade fluorescent contaminants, the autocorrelation peak disappears completely, indicating it is not an instrumental artifact. The width of the autocorrelation peak is a measure of the time a dye molecule stays in the laser beam. The observed peak indicates an effective residence time of the order of 10 ms, which agrees with the value estimated above.

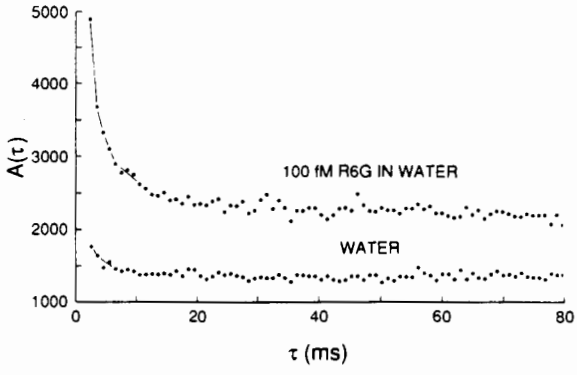

Fig. 3. Autocorrelation spectra for water, with and without addition of 100 fM R6G. The laser power was 30 mW and the autocorrelation was computed over 132 s of data in both cases. The $A(0)$ points are offscale.

The autocorrelation functions shown in fig. 3, which are computed over the passage time of many molecules, indicate that a high sensitivity has been achieved. However, this type of analysis is not able to identify *individual* molecules *as they pass by* – an essential requirement of many potential applications of these techniques.

To examine our data for the passage of individual molecules we have defined a weighted quadratic summing (WQS) filter, given by

$$S(t)=\sum_{\tau=0}^{k-1} w(\tau)\,d(t+\tau)^2\,. \qquad (2)$$

The range k covers a time interval of the order of the molecular passage time; in the present work the value $k=5$ (corresponding to 20 ms) was used. The weights $w(\tau)$ are chosen to best distinguish the signal from passing molecules from random fluctuations in the

background. We chose these weights based on a computer simulation of the expected signal. Typically, the signal increases slowly as the molecule approaches the laser beam, followed by an abrupt cessation when photobleaching occurs. We therefore chose $w(\tau)$ as an asymmetric triangular ramp: $w(\tau) = (\tau+1)/k$ for $\tau=0$ to $k-1$, and $w(\tau)=0$ otherwise. The quadratic form of the WQS produces numerical output that can be readily processed by a threshold discriminator or a peak detector to indicate the presence of a molecule.

The WQS function is a sensitive indicator of the bursts of photons that occur as a molecule passes. In fig. 4 we have plotted values of $S(t)$ for water, with and without added R6G at a concentration of 100 fM. At this R6G concentration approximately one molecule per second passes through the central detection volume. (Only about once in 100 s do two or more dye molecules pass simultaneously.)

The signal amplitude from individual molecules can be expected to vary widely depending upon the path of the molecule through the nonuniform (Gaussian) laser beam and because of statistical fluctuations in the small number of photons that we

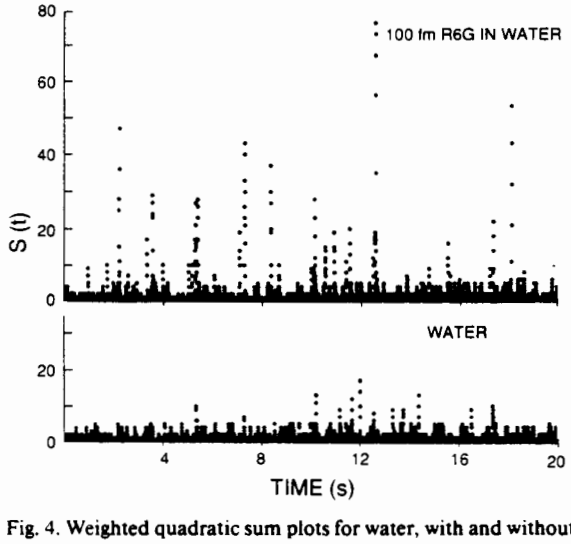

Fig. 4. Weighted quadratic sum plots for water, with and without the addition of 100 fM R6G. In the upper plot, the peaks due to the passage of individual dye molecules are clearly evident. At this dye concentration approximately 1 dye molecule per second passes through the central detection volume. A threshold discriminator set at $S(t)=16$ identifies the passing of the majority of the individual dye molecules.

detect. Many molecules will traverse the fringe of the laser beam and will produce only a weak signal. This effect can be reduced by injecting the sample solution into a rapidly moving surrounding solvent sheath (hydrodynamic focusing) so as to confine the dye to a stream that is smaller in diameter than the laser beam (see ref. [2]).

The data for R6G in water clearly show the occurrence of strong bursts of photons that are almost totally absent when R6G is not added to the solution. (Obtaining solvents totally devoid of fluorescent contaminants is non-trivial since the detection of potential contamination at the level seen here essentially requires a single molecule detection capability, see e.g. ref. [7].) The rate of occurrence of the more intense photon bursts is consistent with the number of molecules per second that pass through the central detection volume. A "molecule detection" threshold discriminator that monitors the WQS-processed data stream readily records the majority of the dye molecules that pass through the central detection volume with only a small chance of false triggers caused by background.

Because the distribution law for the number of photons emitted by a dye molecule before photobleaching is a decreasing exponential, some fraction of molecules will bleach after only a few excitations; these molecules may escape detection. Decreasing the discrimination threshold improves the chances of detecting dim molecules at the price of a higher false rate from statistical fluctuations of the background. At a detection efficiency of 70% (of molecules passing through the central detection volume) our false background rate is less than 0.01 per second, and increases to 0.02 per second when the threshold is lowered to increase the detection efficiency to 85%. If we use a simple sliding-sum filter in place the WQS the error rate is approximately five times higher.

For comparison with the water data, the lower part of fig. 5 shows $S(t)$ for a data stream taken with 100 fM R6G in ethanol. Clearly, ethanol produces a much stronger signal than water. Under the conditions of our experiment, we typically detect bursts of 10–15 photons from R6G in water, whereas from R6G in ethanol we observe bursts totalling 80–120 photons per passing molecule. The more intense bursts seen from R6G in ethanol can be attributed to higher quantum yield and much greater photostability [5]

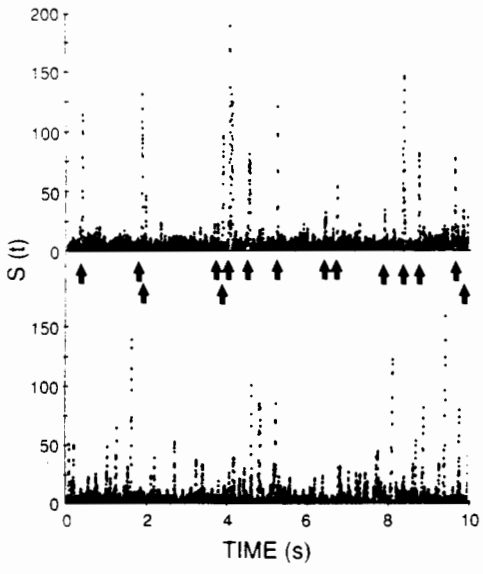

Fig. 5. Weighted quadratic sum plots for 100 fM R6G in ethanol (lower plot) and for a Monte Carlo simulation of same (upper plot). The arrows beneath the upper plot indicate the times when dye molecules passed within the central detection volume during the computer simulation.

of the dye in ethanol as compared with water. Photostability is a key factor in the detectability of single molecules since it directly determines the number of times a dye can be recycled.

To model the expected signal we have developed a Monte Carlo simulation that uses the actual geometry, flow conditions and detection efficiency of our experiment. The simulation, which includes dye saturation due to depletion of the ground state during pulsed laser excitation, the Gaussian intensity profile of the focused beam, Brownian diffusion, and photodegradation and triplet-state lifetime effects, has proven essential for understanding and optimizing the conditions of the experiment. Data generated by the simulation for R6G in ethanol were processed by the WQS filter of eq. (2) for comparison with our actual observations. The results are shown in the upper section of fig. 5. The occurrence of prominent peaks in the simulated data when (simulated) molecules were passing is strong confirmation that peaks in the lower plot correspond to the passage of individual dye molecules through our apparatus. Similar good agreement between experiment and simulation was obtained for R6G in water.

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References

[1] J.H. Jett, R.A. Keller, J.C. Martin, B.L. Marrone, R.K. Moyzis, R.L. Ratliff, N.K. Seitzinger, E.B. Shera and C.C. Stewart, J. Biomol. Struct. Dynam. 7 (1989) 301.
[2] N.J. Dovichi, J.C. Martin, J.H. Jett, M. Trkula and R.A. Keller, Anal. Chem. 56 (1984) 348.
[3] D.C. Nguyen, R.A. Keller and M. Trkula, J. Opt. Soc. Am. B 4 (1987) 138.
[4] K. Peck, L. Stryer, A.N. Glazer and R.A. Mathies, Proc. Natl. Acad. Sci. US 86 (1989) 4087.
[5] S. Soper et al., to be published.
[6] B.J. Berne and R. Pecora, Dynamic light scattering (Wiley, New York, 1976).
[7] T.G. Matthews and F.E. Lytle, Anal. Chem. 51 (1979) 583.