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Single-Molecule Detection Limits in Levitated Microdroplets

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Laser-excited fluorescence from electrodynamically levitated microdroplets is used to detect small numbers of rhodamine-6G molecules. The small sample volume, typically a few picoliters reduces the background due to solvent and impurity Raman and fluorescence emission. With 514.5-nm excitation from an argon ion laser, as few as 12 molecules have been detected in glycerol-water droplets. Our present detection limit, due to variations in the impurity concentration in the blanks, corresponds to a signal-to-noise ratio of 3 for a single molecule of rhodamine-6G contained in a 1-pL volume (droplet diameter of $\simeq 12$ μm).

The two objectives of chemical analysis are the identification and quantification of the species in a sample. The ultimate limit of this objective is to sort each molecule present in the sample, identify it chemically, and tally its number in a log. Although this ultimate approach to chemical analysis is a very ambitious task in general, it appears feasible to apply this concept in a more limited sense if a single species can be selectively detected with near-unit probability. Achieving the ultimate limit of detection, single species, is undoubtedly important to many areas of science. Gas-phase detection of single atoms by resonance ionization and fluorescence spectroscopy (*1*) was demonstrated several years ago. Single molecules have been detected in solids at low temperatures by Moerner and Kador (*2*) and more recently by Orrit and Bernard (*3*). Detection of single molecules in liquid solution at room temperature presents many new challenges and applications.

Hirschfeld first reported the detection of a single polymer molecule (MW 20000) that was tagged with 80-100 fluorescein isothiocyanate molecules and then bound to a single γ-globulin (*4*). Later, Dovichi et al. presented an approach to the detection of single fluorescing molecules in flowing samples (*5*). Their approach involved the use of a sheath flow cuvette to hydrodynamically focus the sample solution into a narrow stream. With the location of the fluorescing species well defined, the sample molecules can pass through a probe volume of a few picoliters (pL). Fluorescence analysis is typically limited to a minimum concentration of 10^{-12}-10^{-13} M due to solvent background, i.e., Raman scattering and fluorescence. Detection of a small number of molecules can be achieved at this concentration by reducing the analysis volume. This approach has made it possible to observe as few as 800 rhodamine-6G (R-6G) molecules (*6*). In an attempt to minimize solvent background, Kirsch evaporated samples on 10-μm silica spheres and observed them with a fluorescence microscope (*7*). This technique allowed the detection of 8000 R-6G molecules. A survey of flow cytometer users has shown that almost one-third of the instruments had a sensitivity of 500 fluorescein-equivalent flurophores per cell (*8*). Nguyen and Keller (*9*) and Peck et al. (*10*) have reported single-molecule detection in the condensed phase by using the protein molecule B-phycoerythrin. This molecule has a molecular weight of 250000 and a fluorescence yield equivalent to ≈25 R-6G molecules (*9*).

Although single-molecule detection has been demonstrated in the experiments of Hirschfeld and with B-phycoerythrin, further improvements in sensitivity are required to detect single molecules of less "efficient" fluorophores. The ability

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to detect single molecules of more conventional fluorophores in solution, such as those used as biomolecular probes or tracers, will greatly increase the applications of fluorescence single-molecule detection. Shera et al. have recently reported that they can detect single R-6G molecules that pass through a laser beam focused into a flow cell (*11*).

In what follows we take a new approach to single-molecule detection in a solution. The development of methods for producing and confining microdroplets for spectroscopy (*12*) gives one the advantages of an extremely small sample volume and immobilization of the sample. Each has its benefit. In a minute volume, the ultimate limit in analyte number (i.e., a single molecule) can still represent a substantial molar concentration. Background effects due to unwanted impurities and Raman scattering fall off rapidly as the droplet shrinks in size. For example, a droplet with a 7-μm diameter has a volume of 0.25 pL, so that one molecule encased in this volume represents a concentration of 10^{-11} M, well above the concentrations that have been previously prepared for single-molecule detection. When a single fluorescent molecule is immobilized in such a trapped microdroplet, the number of absorption–emission events is limited only by photolysis. For R-6G in solution the number of excitations can be in excess of 10^6 (*13*).

The optical properties of microdroplets introduce some potential complications that are not encountered with bulk measurements. A micrometer-sized particle has a large cross section for elastic scattering, and this scattering must be isolated from the fluorescence. However in the case of a spherical droplet, the scattering is highly polarized and predictably anisotropic (*14*) while the fluorescence is almost isotropic and far less polarized (*15*, *16*). Furthermore, a significant portion of the fluorescence is at longer wavelengths than that of the excitation. Consequently, background signal due to elastic scattering can be attenuated to a reasonable level by positioning the detector at a minimum in the angular scattering pattern, using polarized excitation, and by spectral selection of the detected light. We also point out that the internal field of the exciting radiation can be highly anisotropic so that a molecule within the droplet can encounter regions in which the intensity can vary by orders of magnitude (*17*). The spatial intensity variations are much reduced when the pump laser is not tuned to a Mie resonance. These effects will be partially averaged out over the measurement period by rotational diffusion of the droplet and diffusion of the fluorophore within the droplet. The intensity variations result in a variation in the time required to photolyze a molecule (extract maximum signal) but will not prohibit detection of a molecule located anywhere within a droplet.

To obtain an estimate of the actual number of analyte molecules present in a given droplet, we must know the droplet size as well as the concentration. The volume of a droplet can be determined to very high precision (a part in 10^4 has been reported (*18*)) by measuring elastic scattered light as the droplet evaporates. The time dependence of the scattered light shows prominent features due to so-called morphology-dependent resonances (MDR's) (*19*). A spherical particle of radius a irradiated by a plane wave of wavelength λ exhibits an enhanced internal field when its optical size $X = 2\pi a/\lambda$ corresponds to a resonance condition. Each resonance is distinguished by its polarization P (TE or TM for transverse electric and transverse magnetic modes, respectively), angular momentum l, and radial order number s (i.e., the number of nodes of the optical electric field inside the particle). A general mode is labeled $P_{l,s}$. An evaporating particle irradiated at fixed wavelength continuously changes its X value and consequently it "tunes" itself in and out of resonance with time. The resonances appear in the luminescence vs time graph as a series

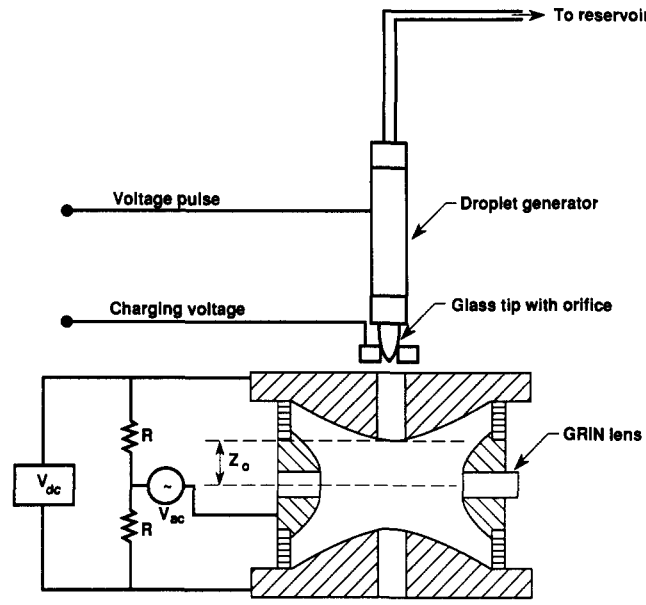

Figure 1. Diagram of hyperbolic electrodynamic trap, droplet generator, and induction charging electrode.

of Lorentzian-shaped peaks. The functional dependence of the elastic scattered light is more complicated, but resonances are easily discernable. Since the X's for each of the modes can be calculated to high precision, one need only identify P, l, and s for a given resonance to obtain the size of the particle. The modes can be identified by comparing the measured time dependence of the elastic scattering with the calculated scattering versus size parameter X from Mie theory (*18*). In addition, unlike fluorescence that radiates in a fairly isotropic fashion, the angular dependence of elastic scattering is particularly sensitive to the polarization of a mode. For example, TM modes do not radiate perpendicular to the plane containing the incident laser beam and polarization vector. Thus a detector placed in this direction will record only transverse electric resonances. The final determination of l and s for a given mode is made through a detailed comparison of the size dependence with Mie theory.

EXPERIMENTAL SECTION

The fluorometric apparatus evolved from a spectrometer described by Arnold and Folan (*20*). Single droplets are suspended in an electrodynamic trap as described by Wuerker et al. (*21*) and later by Frickel et al. (*22*) and Davis and Ray (*23*). A drawing of the trap we used for the present measurements is shown in Figure 1. The three electrodes were machined from aluminum. The hyperbolic surfaces are described by $z^2 = r^2/2 + z_0^2$ for the end caps and $z^2 = r^2/2 - z_0^2$ for the ring, with $z_0 = 0.508$ cm. Holes 0.318 cm in diameter were drilled through the ring electrode at 0 and 90° for optical access. An axial hole of the same diameter in the top and bottom electrodes is used to introduce the droplets into the trap and for supplementary Mie scattering measurements. The electrodes are fastened together with Nylon screws and Teflon spacer rings and mounted in a cylindrical housing, as shown in Figure 2. Voltages of 0–50 V (dc) and 0–1200 VRMS, 60 Hz, are applied to the electrodes as described in ref 20.

The optical arrangement is shown in Figure 2. The excitation laser beam passes through Brewster windows mounted on extension tubes to minimize stray scattered light and reflections. The horizontally polarized beam is focused by a 1-m lens to produce a waist of about 300 μm at the droplet position. The present measurements were made with an argon ion laser at 514 nm with a power at the droplet of 100 mW. Fluorescence photons are collected by a 0.3 cm diameter GRIN lens with 0.25 pitch flush with the interior surface of the ring electrode and a 20X microscope objective and focused through a 0.15 cm aperture spatial filter. The optical speed of the collection optics is approximately $f/2.4$. The fluorescence is spectrally filtered by an interference

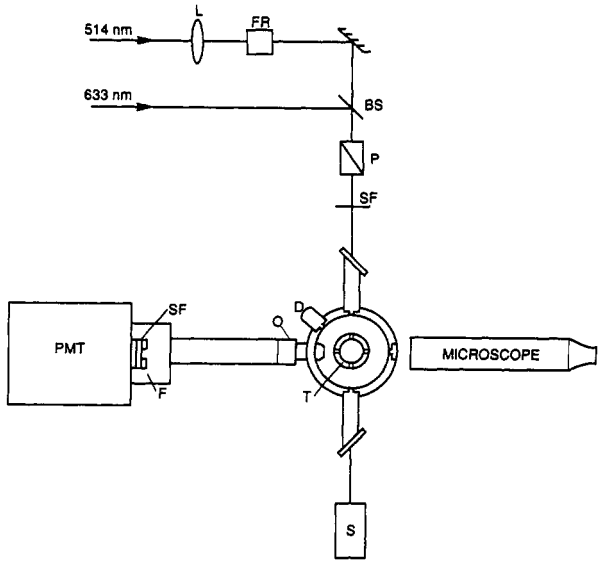

Figure 2. Experimental arrangement for measuring droplet fluorescence: 1-m lens, L; fresnel-rhomb pair, FR; beam splitter, BS; polarizer, P; spatial filter, SF; trap, T; dessicant chamber, D; microscope objective, O; optical filter stack, F; photomultiplier and cooled housing, PMT; and beam stop, S.

filter of 26-nm bandwidth centered at 575 nm and a Corning 3-66 glass filter. The photons are detected by a cooled C31034 photomultiplier, preamplifier, and discriminator–counter. Elastic scattering from the levitated droplets can be observed visually through a window opposite the GRIN lens. The beam from a HeNe laser spatially coincident with the argon laser beam is used to illuminate the droplet without photolyzing the analyte. A microscope with a 2X objective and 20X eyepiece is used to observe the droplets while being trapped. For measurement of droplet diameter, the microscope barrel is extended to increase the effective magnification of the objective to 12.3X. The observation window is blocked during fluorescence measurements to reduce the background signal. A light pipe in the removable lid of the measurement chamber leads to a 3-od neutral density filter and 1P28 photomultiplier tube for measurements of Mie scattering from the droplet (collected with $f/7.6$ optics). The Mie scattering versus time, when measured concurrently with the fluorescence permits an accurate determination of droplet diameter, as discussed below.

Micron-sized droplets are generated by a piezoelectric pipet (*20*). The droplet generator is a commercial unit with a nominal 40-μm hole in the glass tip. In a typical operation, a small quantity of sample or blank solution is sucked into the tip. Droplets are dispensed when a voltage pulse from the power supply constricts PZT piezoelectric strips within the generator head. The diameter of the droplets is about that of the tip orifice when the experiment is conducted near the minimum pulse amplitude. At higher pulse voltages, multiple droplets with smaller diameters can be generated. The droplets are inductively charged by a voltage applied to a copper foil ring supported over the hole in the upper trap electrode.

An accurate value of the volume of the trapped droplets is required to calculate the number of analyte molecules being observed. We obtain an estimate of the droplet diameter from a microscopic examination of the droplet at 90° from the laser excitation. Ashkin and Dziedzic (*24*) have observed that the pattern of scattered light consists of two main spots, one a nonresonant reflected ray scattered from the face of the sphere, the other a nonresonant refracted ray. The former spot will be a distance $(\sqrt{2}/2)a$ from the center of the sphere of radius a, the latter approximately a distance a in the opposite direction, for a total separation of $(1 + \sqrt{2}/2)a$. The microscope body is extended for these measurements to give a greater magnification. The spot separation is measured on an eyepiece reticle with 10-μm rulings that correspond to approximately 1 μm in droplet diameter. The accuracy of visual diameter determination is estimated to be 10%.

Far greater precision in droplet diameter can be obtained by measuring the position of structural resonances in Mie scattering either as the laser frequency is scanned or, in the present experiment, as the droplet evaporates, since the laser frequency is fixed. In the latter case, it is necessary to work with the relative positions of resonances of different order, assisted by an estimated diameter from the visual observations. Once the resonances have been identified, the size parameter, and hence the diameter if the refractive index of the droplet is known, can be determined to about one part in 400, limited by our time resolution and the uniformity of the evaporation rate. In practice, experimental scattering curves are matched with Mie scattering calculations. A Fortran program given by Bohren and Huffman (*14*, *18*) is used for this purpose. The program has been modified so that the output represents an average over the appropriate range of azimuthal angles.

The analyte for the present investigation was rhodamine-590 chloride laser dye from Exciton, as obtained from the manufacturer. The R-6G was first diluted to 4.17×10^{-9} M in semiconductor grade methanol, Alfa Products No. 19393, and then further diluted in a mixture of water and glycerol, in a ratio of 9:1. The water was HPLC grade, Baker No. 4218-03, the glycerol was E M Science No. GX0187-3. The solvents were not further purified. Blanks were made by mixing 9:1 proportions of water and glycerol.

In a typical measurement, the argon laser beam is blocked and the HeNe laser turned on. A droplet of the desired solution or blank is suspended in the trap. The solvent concentration in the droplet approaches steady state in a few seconds. While the droplet is visually observed through the low-power microscope, the dc voltage is adjusted to center the droplet and an ac voltage that will maintain particle stability (*22*, *25*) is selected. The HeNe laser is then turned off, the observation window blocked, the photon counters initiated, and the argon laser beam admitted to the chamber. With 100 mW of laser power, the detected emission from the sample or blank decays in about 20 s to background. Elastic scattered photons are recorded in a second channel of the photon counter for droplet size determination. Data are typically recorded for 2000 s, during which time the optical size, X, of the droplet decreases by 4 or 5 units. At the conclusion of the measurement, a visual determination of droplet diameter is made by measuring the separation of the reflected and refracted spots with the higher power microscope.

RESULTS

We present first the results of some fluorescence and elastic scattering measurements made at high R-6G concentration, 5 μM, and low laser intensity, 100 mW cm^{-2}, so that most of the R-6G remains for the duration of the experiment. The fluorescence as a function of time from a 10-μm droplet is shown as the lower curve in Figure 3. The curve shows numerous features of varying widths corresponding to structural optical resonances of the droplet. The curve above shows the elastic scattering from the droplet in the vertical direction, measured at the same time. As discussed above, the resonances observed in this coherent elastic scattering are predominantly TE resonances, while the fluorescence excitation shows both TE and TM resonances. The upper curve is a theoretical calculation of the droplet absorption versus size parameter ($2\pi a/\lambda$, top scale), with an assumed refractive index of $1.47 + i0.00002$. The mode assignments were determined by the procedures described earlier. At a given droplet size, only two different mode orders are readily discernable for either TE or TM modes. The third curve from the bottom is the calculated Mie scattering integrated over a 9° range of azimuthal angle. The qualitatively good agreement between the calculated and experimental curves shows that the particle diameter obtained with the microscopic measurements is correct to within 1 μm.

Our determinations of detection limit were made with a laser intensity of 310 W cm^{-2}, sufficient to photolyze the analyte molecules in a few seconds. A typical experimental record is shown in Figure 4. The sample was a 13-μm droplet of 17 pM concentration of R-6G in glycerol–water. The lower

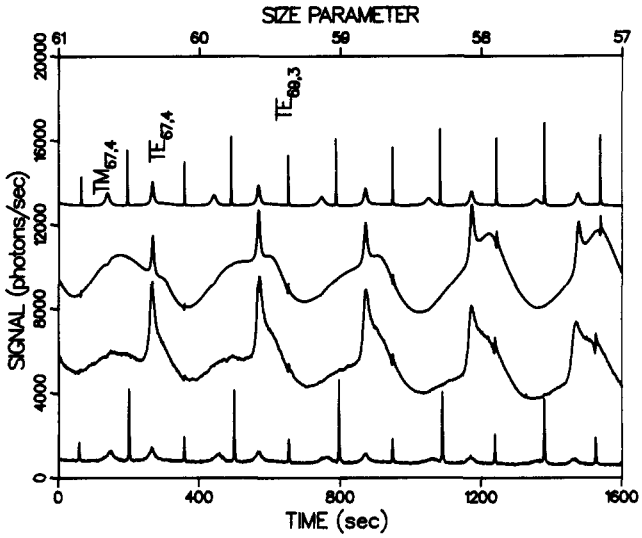

Figure 3. Experimental and calculated curves of droplet fluorescence excitation and elastic scattering vs time for a 10-μm droplet with 5 μM R-6G: (from bottom) experimental fluorescence excitation, experimental elastic scattering, theoretical elastic scattering, theoretical fluorescence excitation.

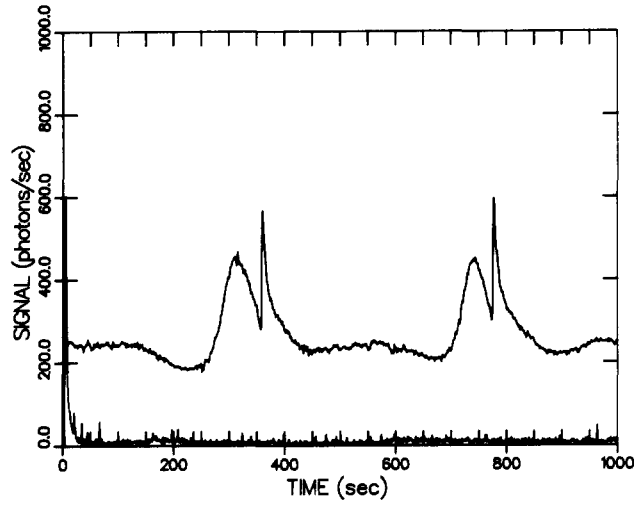

Figure 4. Fluorescence (lower curve) and elastic scattering vs time from 13-μm droplet with 17 pM R-6G concentration.

curve is the detected fluorescence as a function of time after the laser beam is unblocked. Most of the signal decays in a few seconds as the R-6G is photolyzed. The signal remaining after about 20 s is believed to be mainly fluorescence from the filters or the black paint in the trap excited by the elastic scattering from the droplet. The latter contribution is more pronounced at the broad structural resonances near 200 and 600 s. The upper curve is the elastic scattering measured in the vertical direction as the droplet slowly evaporates. The incident laser beam is horizontally polarized and propagating horizontally. The structural resonances in this scattered light are excited by light polarized perpendicular to the scattering plane and are therefore transverse electric (TE) modes (*14*). The broad resonances observed in the fluorescence background are transverse magnetic (TM). The higher laser intensities within the droplet at either TE or TM resonances will produce corresponding features in the incoherent fluorescence and Raman emission. Some of the sharp features in the background may be of this nature. We also observe occasional count spikes even with the photomultiplier blocked, possibly due to cosmic ray scintillations or electrical noise. It is possible to discriminate between structural resonances and these spurious peaks by reducing the counting time per point. The

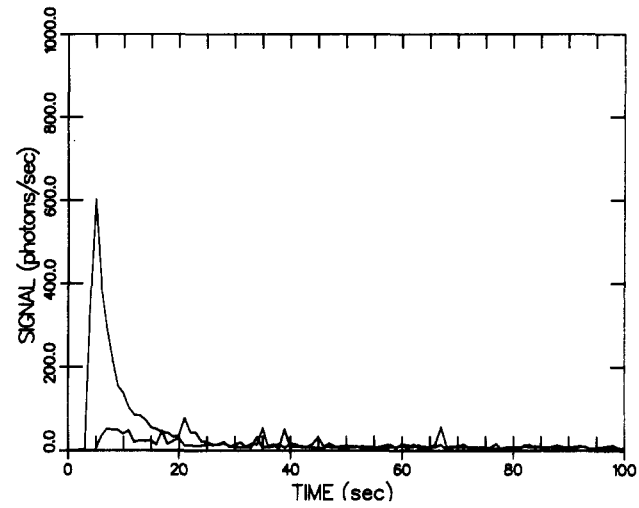

Figure 5. Fluorescence vs time for 13-μm sample and blank droplets. Upper curve, 17 pM sample; lower curve, blank.

Table I. Results for Blank, 17-pM Droplets

no. of net photons counted in 20 s	droplet vol, pL	no. of R-6G molecules from conc	no. of R-6G molecules from counts
382	1.2	0	1.3
1908	11.5	0	6.6
669	4.2	0	2.3
11295	2.6	26	37
2630	1.2	12	8
8451	3.0	31	27

sharpest resonances we can observe have a duration of several tenths of a second under our evaporation conditions, while the noise events occur in less than 0.1 s.

A plot of the same fluorescence data with expanded time scale is shown in Figure 5 together with similar measurements on a droplet of pure solvent, also of 13-μm diameter. The initial fluorescence from the blank droplet shows a photolyzable component presumably due to impurities in the solvent. Variations in the size of this component from droplet to droplet limit the ultimate detection we can achieve.

In Table I we present the results of six measurements, three for blank droplets (no R-6G added) and three for droplets with 17 pM concentration of R-6G in glycerol. The photocounts were integrated over the initial 20 s of exposure to the argon laser beam with background counts over a similar but much later time period subtracted. The expected number of R-6G molecules in each sample droplet is calculated from the concentration and the measured diameter. All water is assumed to have evaporated. (At 50% relative humidity, the droplets should contain about 20% by volume of water according to Raoult's law (*26*). We are therefore overestimating the number of molecules present in the droplets.) The three blank samples gave an average of 214 (±90) counts pL^{-1} over background during the 20-s period. This quantity was calculated for each droplet and then averaged to avoid overweighting the larger drops. If this value is subtracted from the counts with R-6G added, again for each droplet, one obtains an average for the three samples of 288 (±112) counts/R-6G molecule. Dividing this number into the net photons counted gives the three values for the last column in the table when R-6G is added. The equivalent concentration of R-6G molecules in the blank droplets obtained from the above calculated average of 288 counts/R-6G molecule would be 0.7 R-6G pL^{-1} or 1.2 pM. Twice the noise in the integrated blank photocounts is 180 counts pL^{-1}, equivalent to 0.6 R-6G pL^{-1}. This value can be taken to be our present detection limit, with the main source

of noise being photolyzable impurities in the solvents.

DISCUSSION

The results presented above show that fluorescence measurements on microdroplets are extremely sensitive and that detection of a single R-6G molecule or its equivalent is possible by this technique. We are at present limited only by solvent purity and reproducibility of the blank determinations. Even so, our estimated signal-to-noise ratio of 3/R-6G indicates that we should be able to detect single molecules of small fluorophores with the microdroplet technique.

Within the framework of the present method, the detection limit could be substantially improved in a number of ways. Most of the background signal, both photolyzable and permanent, is proportional to the volume of the droplets. Thus, impurity fluorescence and solvent Raman scattering would be reduced if smaller droplets could be measured. Indirect background, such as fluorescence excited by elastic scattering from the droplets, varies as the cross sectional area for micrometer-sized droplets and therefore would also be reduced for smaller droplets. An even larger advantage can be obtained for submicrometer particles. Rayleigh scattering from particles with $a \ll \lambda$ falls off as a^6 (*14*). Elastic scattering and fluorescence of the particle trap directly excited by the laser are the main sources of background that are independent of particle size. These are readily measured with no particle present in the trap to be ~ 5 counts s^{-1}, or a noise equivalent of 0.06 R-6G molecules.

The departure from the expected number of R-6G molecules calculated from the concentration is given by the difference between the numbers in the second and third columns in Table I for the three measurements with R-6G present. If we neglect possible errors in volume determination and dilutions, the values are entirely consistent with a Poisson distribution for the number of molecules in a microdroplet. Further statistical studies using the present method are desirable. These would be easier to interpret if the droplets used were monodisperse. The dispersity in volume per droplet in the present study results from selecting satellite droplets to obtain small diameters. Lin et al. (*27*) have recently reported modifications to a Berglund and Liu generator (*28*) that reduce size fluctuations to less than ±3 parts in 10^4 for droplets in the 10–80-μm range.

Measurements of fluorescence of molecules confined to small droplets permit the determination of photochemical lifetimes in two independent ways. One way makes use of the total number of photocounts detected from a collection of molecules. When divided by the absolute collection efficiency of the photon counting system and the number of analyte molecules, the number of photoexcitations before photolysis is obtained. An alternative determination can be obtained from the fluorescence decay time, the calculated laser intensity within the droplet, and the molecular extinction coefficient. With the former method and estimates of our collection efficiency, we obtain a lifetime for R-6G of from 10^5 to 10^6 cycles, in good agreement with the value of 2×10^6 from Ippen et al. (*13*).

Registry No. R-6G, 989-38-8.

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