Digital Chemical Analysis of Dilute Microdroplets

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The concept of digital molecular detection, the ability to count individual molecules, is described and the mathematical framework for defining detection limits under various experimental constraints presented. We show that with modest signal-to-noise ratio for detection of single molecules, significant advantages accrue when using a digital detection strategy versus a conventional approach. Concentration detection limits decrease inversely with total sample volume for digital detection versus reduction as the inverse square root for conventional measurements. This advantage could yield detection limits that are reduced by orders of magnitude if sample volume is limited. In addition, we present experimental results demonstrating the concept of digital molecular detection using β-phycoerythrin molecules and levitated microdroplet fluorimetry. Single β-phycoerythrin molecules are detected with a signal-to-noise ratio greater than 4.

INTRODUCTION

The mass limit of detection of molecules in solution by laser-induced fluorescence has been steadily reduced (fewer molecules) in recent years, primarily by decreasing the volume of solution that is probed.[1-7] The reduction has been achieved in some cases by optically restricting the excitation volume and/or the volume observed by the detector, by decreasing the size of the sample in one or more dimensions, or by a combination of these techniques. The signal-to-noise ratio is also enhanced if the analyte molecules remain in the measurement volume for a large number of excitation–emission cycles, ideally until they photolyze.[4] Several experiments have been reported in which the sensitivities are sufficient to detect single molecules.[4-7] The approach we have taken is to use levitated microdroplets as the sampled medium.[6] This approach has several important features: the measurement volume (of the order of 1 pL) is defined by the droplet, the analyte is confined to the droplets, and time is an independent variable so that the fluorescent molecules present in a droplet can be observed for a time longer than their photochemical lifetime. Thus, any and all analyte molecules present should be detected if the signal-to-noise ratio is sufficient. When the concentration of analyte molecules is in the picomolar range, at most a few analyte molecules will be present in a given droplet, with the probability governed by Poisson statistics. With certain dye molecules and very dilute solutions, we are now able to count the analyte molecules in a microdroplet. That is, the number of detected fluorescence counts per analyte molecule is sufficiently greater than the fluctuations in the background counts over an equal time interval that we can say with reasonable certainty whether or not a molecule of interest is present. We are therefore in effect performing a digital analysis.

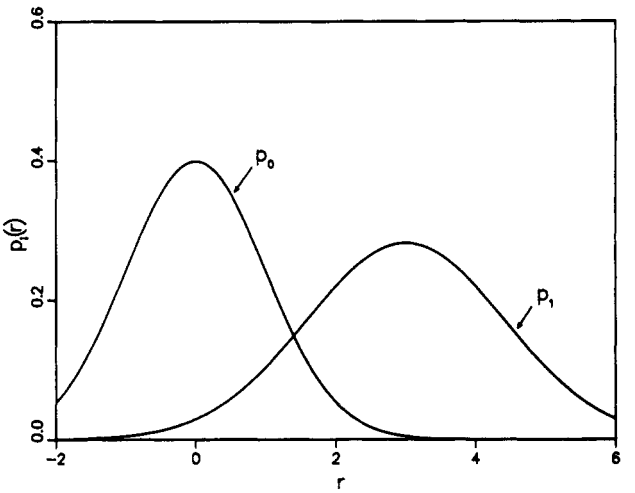

Figure 1. Probability distributions for a typical measurement. The quantity $p_0(r)\,dr$ is the probability that a blank will produce a measured signal with a value between r and $r + dr$; $p_1(r)\,dr$ is the probability for a droplet with an analyte molecule present.

In this paper we show theoretically that concentration detection limits can be lower using a digital detection strategy (counting molecules) versus the conventional approach where both signal and background are integrated. This concept was recognized by Peck et al.,[4] and their results indeed demonstrated that the detection limit could be improved by counting individual molecules. Stevenson and Winefordner[8] have defined the requirements for single atom/molecule detection in terms of detection efficiency and the probability of false negative declarations. They point out the importance of a well-defined probe volume. Various statistical tools can be employed to decide if a single analyte molecule is present or to estimate the number in case there should be more than one. The determinations are constrained, however, to measurements within the photolytic lifetime of the analyte molecule unless the photolysis process yields a product that can be detected by the fluorescence spectrometer.

Below, we first show that the statistical methods classified as hypothesis testing can be used to optimize a digital molecular detection strategy given a priori knowledge of some experimental parameters. This theory is further developed to predict concentration detection limits given photocount distribution functions for background and fluorescence signals. It is shown that the improvement in detection limit

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(2) Nguyen, D. C.; Keller, R. A.; Trkula, M. J. *J. Opt. Soc. Am.* **1987**, *B4*, 138–143.
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(5) Shera, E. B.; Seitzinger, N. K.; Davis, L. M.; Keller, R. A.; Soper, S. A. *Chem. Phys. Lett.* **1990**, *174*, 553–557.
(6) Whitten, W. B.; Ramsey, J. M.; Arnold, S.; Bronk, B. V. *Anal. Chem.* **1991**, *63*, 1027–1031.
(7) Hahn, J. H.; Soper, S. A.; Nutter, H. L.; Martin, J. C.; Jett, J. H.; Keller, R. A. *Appl. Spectrosc.* **1991**, *45*, 743–746.
(8) Stevenson, C. L.; Winefordner, J. D. *Appl. Spectrosc.* **1992**, *46*, 407–419.

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that can be achieved by a digital detection strategy depends on the statistical distribution of the noise as well as on the magnitude of the signal, background, and the signal and background noise. In addition, experimental results are presented that demonstrate the detection of single molecules of β-phycoerythrin in levitated microdroplets with a signal-to-noise ratio greater than 4. These experiments show the feasibility of implementing a digital detection strategy for highly fluorescing molecules.

THEORY

Let us first consider a measurement of a solution sufficiently dilute that the probability of finding two molecules in a microdroplet is inconsequential. Mathematical methods for detecting the presence of a single molecule fall into the category of hypothesis testing,[9] with the two hypotheses being in the present case the presence or absence of an analyte molecule. The outcome of a given measurement is typically the number of photocounts obtained in a well-defined time period. The counts will consist of signal and noise. The space of all possible measurement outcomes is divided into two regions, one mandating the choice of a blank, the other to declaring a detected molecule. Where this division occurs depends not only on the statistical properties of the signal and noise, but also on a threshold criterion that can be selected by the priorities of the experiment. For example, if the presence of extremely dangerous molecules is being detected, missing a molecule is probably far more costly than a false alarm so the detection threshold could be adjusted accordingly.

There are a number of different ways to choose a decision strategy that best satisfies the goal of the experiment, depending on the information already known about the sample and the relative importance of the possible errors. Let $p_0(r)\,dr$ be the probability distribution of the result, r, when the sample is a blank, i.e., of the background noise, and $p_1(r)\,dr$ be the distribution when an analyte molecule is present, signal plus noise. The variable r would correspond to the number of detected photons in our case. The mean background has been subtracted in each case. (See Figure 1 for examples of p_0 and p_1.) $P(0)$ is defined as the probability that the sample is a true blank; $P(1) = 1 - P(0)$, the probability of a molecule being present. $P(0)$ and $P(1)$ depend upon the concentration of the solution and the size of the probe volume. One decision strategy due to Siegert[10] is to declare a molecule when, for a measured photocount, r

$$\frac{p_1(r)}{p_0(r)} \geq \frac{P(0)}{1-P(0)} \tag{1}$$

is satisfied and declare a blank if not. The ratio, $p_1(r)/p_0(r)$ is called the likelihood ratio. The larger this ratio, the smaller the probability error, P_e. This criterion minimizes the probability of error, P_e, given by

$$P_e = P(1|0)P(0) + P(0|1)[1 - P(0)] \tag{2}$$

where $P(0|1)$ is the conditional probability that a blank is declared when a molecule is actually present and $P(1|0)$ the probability of a false positive. The criterion does not take into account the relative importance of these two kinds of errors. It is sometimes called the minimum error probability criterion[11] or the ideal observer test.[10] If the likehood ratio is a monotonic function of r, as when p_0 and p_1 are Gaussian distributions, the test can be performed by comparing r with a threshold, i.e., the value of r that makes eq 1 an equality. More complicated distribution functions may have more than one region where a blank or molecule should be declared.

An example of probability distributions $p_0(r)$ and $p_1(r)$ is shown in Figure 1. Both curves are chosen to be Gaussian with standard deviations of 1 and $\sqrt{2}$, respectively. A single threshold would divide the space r into the two regions in which a blank or detected molecule should be declared. We will not address probability distributions that give likelihood ratios that are not monotonic in r. For those more complicated distributions, the likelihood ratio test, eq 1, could be satisfied in several regions so that a single threshold would not suffice.

A more general detection strategy is to assign a different cost to each of the four possible results of a measurement. We define two more conditional probabilities: $P(0|0)$, the probability that a blank is declared when no molecule is present, and $P(1|1)$, the probability that a molecule is declared when one is present in the sample. The four costs are C_{00}, C_{01}, C_{10}, and C_{11}. The Bayes criterion[12] for a measurement is obtained by minimizing the average cost, given by

$$\bar{C} = P(0)[P(0|0)C_{00} + P(1|0)C_{10}] + [1 - P(0)][P(0|1)C_{01} + P(1|1)C_{11}] \tag{3}$$

In terms of the previous distribution functions, a blank should be declared when

$$\frac{p_1(r)}{p_0(r)} < \frac{P(0)[C_{10} - C_{00}]}{[1 - P(0)][C_{01} - C_{11}]} \tag{4}$$

is satisfied.

Application of either this criterion or the previous one to achieve a true minimum error probability or cost requires an a priori knowledge of $P(0)$, hence the concentration of the analyte in the solution. Since this concentration is usually the desired result of the analysis, these criteria would seem to have only limited analytical utility. However, as shown below, they can be used to find a threshold that gives the optimum error or cost at some chosen concentration, or conversely, to find that minimum concentration (the detection limit) at which the probability of error or cost per molecule is just acceptable. Measurements at higher concentrations with the same threshold will not be optimum but will nevertheless result in a lower error per detected molecule than measurements at the limit of detection because there will be fewer chances for false positive errors.

A different criterion for analysis of dilute solutions that does not require a priori knowledge of the concentration is based on limiting the maximum error for either false positives or false negatives within the constraints of the signal and noise.[9,11,12] For the Neyman–Pearson criterion, the maximum probability of a false positive error, $P(1|0)_{max}$, is chosen based on the experiment. For example, at most one false positive per thousand droplets if the signal-to-noise ratio permits. $P(1|0)_{max}$ can always be made as small as desired while, for non-negative threshold, the same is not true for $P(0|1)_{max}$. A threshold is determined from the background noise distribution function, $p_0(r)\,dr$

$$P(1|0)_{max} = \int_t^\infty p_0(r)\,dr \tag{5}$$

giving for the probability of detection

$$P(1|1) = \int_t^\infty p_1(r)\,dr \tag{6}$$

(9) Helstrom, C. W. *Statistical Theory of Signal Detection*; Pergamon Press: New York, 1960.

(10) Lawson, J. L.; Uhlenbeck, G. E. *Threshold Signals*; McGraw-Hill: New York, 1950.

(11) Whalen, A. D. *Detection of Signals in Noise*; Academic Press: San Diego, 1971.

(12) Neyman, J.; Pearson, E. S. *Phil. Trans. R. Soc.* **1933**, *A231*, 289–337.

Alternatively, the maximum probability of a missed detection, $P(0|1)_{max}$ could be selected and the threshold found from

$$P(0|1)_{max} = \int_{-\infty}^{t} p_1(r)\, dr \tag{7}$$

The maximum possible cost can be minimized with no a priori knowledge of sample and blank probabalities via the minimax criterion.[9,11] The minimax cost found by maximizing eq 3 with respect to $P(0)$ is given by

$$\bar{C} = C_{10}P(1|0) + C_{\infty}P(0|0) = C_{01}P(0|1) + C_{11}P(1|1) \tag{8}$$

If the costs for correct results are set to zero, the minimax condition becomes

$$C_{10}P(1|0) = C_{01}P(0|1) \tag{9}$$

where the conditional probabilities are given by

$$P(1|0) = \int_{t}^{\infty} p_0(r)\, dr \tag{10}$$

and

$$P(0|1) = \int_{-\infty}^{t} p_1(r)\, dr \tag{11}$$

This criterion would appear to be useful mostly when $P(1)$ is of the same order as $P(0)C_{10}/C_{01}$. It is unlikely that $P(1)$ would be so large for the dilute solutions under consideration.

From an analytical viewpoint, it is of interest to work backward from these results. That is, what is the minimum concentration of analyte that can be detected with a given probability of error or cost per molecule detected given the experimental probability distributions for the signal and noise? From Poisson statistics, the blank probability is given by $P(0) = \exp(-n)$ where n is the average number of analyte molecules per sample droplet. An excellent approximation for very dilute solutions, hence $n \ll 1$, is $P(0) = 1 - n$, $P(1) = n$.

We can define a detection limit in terms of the maximum volume of solvent (number of droplets) per analyte molecule that can be measured without exceeding a given error probability. With this concept of detection limit and the dilute samples we are considering, a large number of blanks will be detected for each detected molecule. If we consider a measurement to consist of $1/n$ determinations on droplets consisting on the average of N blanks and one containing an analyte molecule, $N + 1 = 1/n$, then the probability of a single error for the entire measurement, P_{eT}, will be given by the first term in the binomial expansion

$$P_{eT} = \frac{(N+1)!}{1!N!} P_e (1 - P_e)^N \tag{12}$$

with P_e given by eq 2, or approximately

$$P_{eT} \approx (N+1)P_e \tag{13}$$

If we express $P(0)$ and $P(1)$ given above in terms of N rather than n, ($P(0) = N/(N+1)$ and $P(1) = 1/(N+1)$) and substitute these values in eq 2 to get P_e, then eq 13 becomes

$$P_{eT} = NP(1|0) + P(0|1) \tag{14}$$

or

$$N = \frac{P_{eT} - P(0|1)}{P(1|0)} \tag{15}$$

The total cost per detected molecule will in the same spirit be, with $C_{11} = C_{00} = 0$,

$$C_T = NC_{10}P(1|0) + C_{01}P(0|1) \tag{16}$$

For a detection with 95% confidence, for example, we should have $P_{eT} = 0.05$. The conditional probabilities are found from eqs 10 and 11, and the threshold is the value of r that makes the two sides of eq 1 or 4 equal. The molar concentration detection limit would then be $n/VA = 1/(N+1)VA$ where V is the average droplet volume in liters and A is Avogadro's number. Note that for the Bayes criterion the detection limit will be improved in proportion to the relative cost of false positives.

The threshold criteria of the Neyman–Pearson type can be treated the same way, through P_{eT} or C_T though now either $P(0|1)_{max}$ or $P(1|0)_{max}$ is fixed, determining the threshold and the other error and detection probabilities. These criteria do not require an a priori blank probability so the probability of error or cost cannot be estimated in general. However, when we pick a test concentration, the blank and analyte molecule probabilities can then be calculated, permitting an evaluation of P_{eT} or C_T. The expressions for N and the molar concentration detection limit will be the same as above except for the new conditional probabilities.

The probability functions and the thresholds for the various criteria are readily calculated when the noise is Gaussian and the signal is well defined, such as in digital electronic circuits. However, in many cases as in the present investigation, there are non-Gaussian contributions to the background noise and possibly to the signal as well so the signal and noise distribution functions must be determined experimentally by measuring solutions of known concentration. The probability distribution of photocounts for droplets with an analyte molecule present has contributions for the background noise as well as the fluorescence from the analyte. The latter contribution depends on the way the analyte molecule photolyzes. In particular, some molecules contain more than one fluorophore that may photolyze independently. We have shown[13] that the functional form of the signal contribution can be described by a Poisson distribution

$$p_1(r)\, dr = \frac{m}{R}\left(\frac{mr}{R}\right)^{m-1} \frac{e^{-mr/R}}{(m-1)!}\, dr \tag{17}$$

where m is the number of independently photolyzable fluorophores on the molecules and $R = \langle r \rangle$ is the mean number of photocounts per molecule. In this expression, r is the number of emitted rather than detected photons since no provision for sampling statistics has been included, and is assumed to be $\gg 1$.[2,6] An experimentally determined distribution would also be convoluted with the background noise and any additional signal noise, thus more closely resembling the distributions of Figure 1. For single molecules with a single fluorophore, i.e., for $m = 1$, the probability distribution is a decaying exponential. The substantial probability density in the vicinity of $r = 0$ precludes a complete separation of signal and the background noise. Even though the value $r = 0$ is more probable than other values for a single fluorophore, the probability that all fluorophores emit zero photons is vanishingly small. (Note also that $p_1(r)$ for m fluorophores with total mean photocounts R is not m times $p_1(r)$ for 1 fluorophore with average R/m because in the former case all of the combinations of photocounts that give the total result r must be considered.) For m equal to 2 or higher, the density is zero at $r = 0$. Thus, there appears to be a significant advantage in total error for molecules that contain more than one fluorophore even if the mean number of photons emitted is the same as for a single fluorophore. As the number of fluorophores becomes large, the probability distribution approaches a Gaussian distribution.

As an example of the performance that could be expected of digital analysis when the signal and noise distributions are Gaussian, we present the following calculation. Suppose that

(13) Whitten, W. B.; Ramsey, J. M. *Appl. Spectroc.*, in press.

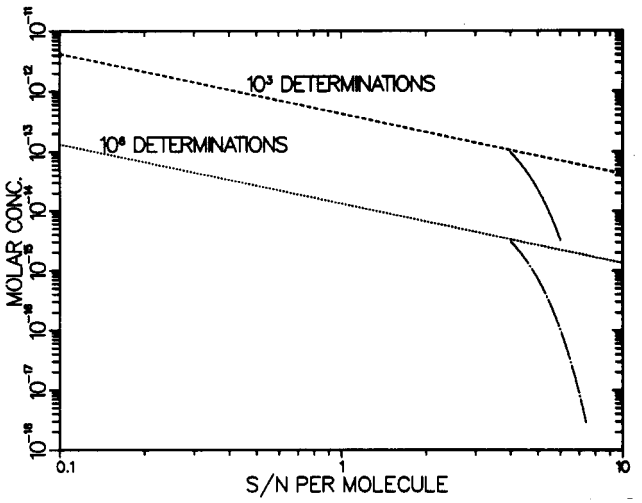

Figure 2. Concentration detection limit as the signal per analyte molecule is varied. The background noise is assumed to be the same for all samples. Measurement volume is 0.5 pL. Signal-to-noise ratio of 4 at a concentration of one molecule per measurement volume is assumed. The dashed and dotted curves represent conventional detection for 10^3 and 10^6 measurements. Solid and chain-dot curves are for digital detection with signal and background noise of equal variance for a total error of 0.05.

measurements on droplets with an average volume of 0.5 pL give a photocount of 500 with a standard deviation of 100 counts when a single molecule is present, with a background standard deviation also of 100 counts. What will be the limit of detection with the minimum error criterion if a total error of 0.05 per detected molecule is expected, or a confidence level of 95%? We have

$$0.05 = \frac{N}{\sqrt{2\pi}}\int_t^\infty \exp[-r^2/(2\times 10^4)]\,dr + \frac{1}{\sqrt{2\pi}}\int_{-\infty}^t \exp[-(r-500)^2/(2\times 10^4)]\,dr \quad (18)$$

and, equating the two sides of eq 1 with $r = t$ and $P(0)/P(1) = N$

$$N = \frac{\exp[-(t-500)^2/(2\times 10^4)]}{\exp[-t^2/(2\times 10^4)]} \quad (19)$$

Solving simultaneously, we find $t = 312$ and $N = 22$, giving a detection limit for 95% confidence of 1.5×10^{-13} M. Measurements at higher concentration will give a lower error per molecule and hence a higher level of confidence even though the error per measurement is no longer optimum. Note that if the 23 droplets were combined and measured simultaneously (for one photochemical lifetime), the noise would be $23^{1/2}$ times larger, but the signal would be unchanged, giving a poorer limit of detection. For a confidence level of 90%, the limit of detection for digital analysis falls to 2.7×10^{-14} M. The detection limit for 95% confidence and an average signal of 600 counts with the same 100 counts of noise would be 3.1×10^{-15} M ($t = 416$, $N = 1060$). Thus, a modest increase in signal-to-noise ratio produces a large improvement in the projected limit of detection.

To achieve improved detection limits by digital detection, the average number of analyte molecules per measurement volume must be less than one. As the ratio of the signal per molecule to the noise of a null droplet increases, the detection limit falls exponentially. We show in Figure 2 a comparison of detection limits obtained by digital and conventional techniques for equal total sample volumes. We have plotted the minimum detectable concentration for 10^3 and 10^6 determinations with a measurement volume of 0.5 pL. For a fixed probe volume, and thus presumably a fixed background noise, the abscissa is then proportional to the number of detected signal photons per molecule. We have chosen the signal-to-noise ratio for one molecule in a 0.5-pL volume to be 4. The dashed curve represents the detection limit obtained conventionally averaging the results of 10^3 determinations; the dotted curve, for 10^6 determinations. Both curves give a linear dependence of detection limit vs signal per analyte molecule. The solid and chain-dot curves show the detection limits that can be obtained with digital detection for equal signal and noise variances and a total detection error of 0.05. This error does not include statistical sampling errors due to the small number of molecules detected. Gaussian photocount distributions have been assumed.

We should point out that for equal total sample volumes, the improved digital detection limits are obtained at the expense of measurement time because the conventional measurements can be made on larger probe volumes. The duration of each determination should be the analyte photolysis lifetime. Conversely, if d determinations are required for the digital measurement, a conventional measurement could achieve the same detection limit in the same total time with d times more sample volume. The power of digital analysis is most apparent when the entire sample is to be analyzed.[1,14] Statistical variations due to sampling are no longer present since all analyte molecules are counted. For adequate signal-to-noise ratio, the limit for digital detection is one analyte molecule per total sample and thus decreases as d^{-1} with d determinations. This detection limit will be $d^{1/2}$ times lower than can be obtained by integrating the signal and background with conventional techniques but at the expense of measurement time. The inverse linear dependence of detection limit with total number of probe volumes has been previously pointed out by Dovichi et al.[1] and by Alkemade.[14]

EXPERIMENTAL SECTION

The experimental apparatus and measurement procedures are the same as reported in ref 6. Electrically charged microdroplets are generated with a piezoelectric pipette and levitated in an electrodynamic trap.[15] The droplets are exposed to the beam from an argon ion laser. The resulting fluorescence is spectrally filtered to remove the elastic and Raman scattering background and then detected with a cooled photon-counting photomultiplier. The present measurements were made with a laser wavelength of 514 nm with an intensity at the center of the beam of 300 W cm^{-2}.

The major differences between this and the previous work are in the samples and their preparation. The molecule β-phycoerythrin was chosen because of its high fluorescence output per molecule[16] and its prior use in single molecule detection research by others.[3,4] Special care was taken in the selection and mixing of solvents and in preparing the dilutions, as described below.

Glycerol (EM, Omnisolve No. GX0187-3) and ultrapure, sterile water (Carolina Biological Supply Co.) were used to prepare the solutions containing β-phycoerythrin (Molecular Probes, Inc. P-800). The concentrations were calculated according to the dilutions in glycerol. Addition of water served to reduce the droplet diameter (volume) after evaporation. The working solutions were prepared daily and diluted with the water; the resultant solutions contained 2% (v/v) glycerol.

All the glassware was cleaned as follows: soaked in Micro (International Products Corp.) laboratory cleaning solution for ~24 h; rinsed several times with distilled water; soaked in ~10 M ultrapure nitric acid for ~24 h; rinsed several times with distilled water, HPLC-grade methanol, semiconductor-grade

(14) Alkemade, C. Th. J. In *Analytical Applications of Lasers*; Piepmeier, E. H., Ed.; Wiley: New York, 1986; pp 107–162.
(15) Arnold, S.; Folan, L. M. *Rev. Sci. Instrum.* **1986**, *57*, 2250–2253.
(16) Mathies, R. A.; Stryer, L. *Applications of Fluorescence in Biomedical Sciences*; Taylor, D. L., Waggoner, A. S., Lanni, F., Murphy, R. F., Birge, R. R., Eds.; Alan R. Liss, Inc.: New York, 1986; pp 129–140.

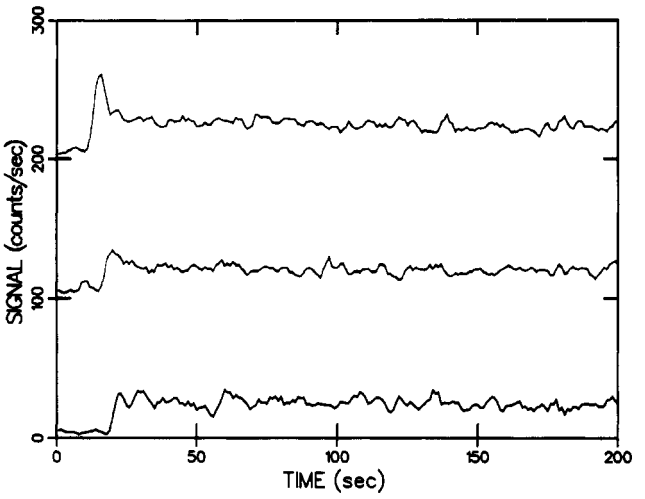

Figure 3. Number of photocounts per second versus time for three different droplets. Upper curve, droplet with one β-phycoerythrin molecule present, diameter 11 μm; middle curve, blank droplet, diameter 10 μm; lower curve, droplet of dilute β-phycoerythrin solution containing no analyte molecules, diameter 10 μm. The two upper curves have been shifted vertically for clarity. The data have been smoothed by a three-point running average.

methanol (Alpha Products No. 19393), and finally several times with the ultrapure sterile water. The glassware was rinsed with a solution of the same concentration before use, and the glassware containing the solution was covered with Parafilm. Disposable pipets and beakers were used for transfer of aliquots and containing the sample solutions, respectively. They had been previously rinsed with semiconductor-grade methanol, ultrapure sterile water, and the solution to be used.

The droplet generator (Uniphoton Systems, Inc.) was rinsed with semiconductor-grade methanol and a blank solution prior to sample introduction. A microliter volume of sample solution was drawn into the tip of the droplet generator by manually reducing the pressure over the reservoir. Between samplings, the previous solution in the tip was discarded. The tip was then rinsed inside and out with semiconductor-grade methanol, ultrapure sterile water, and the solution to be sampled. After the tip was filled with sample solution, the outside was again washed with methanol, and the methanol was allowed to evaporate. The first few droplets from the generator were discarded to minimize the chances of contamination. Care was exercised to prevent the droplet generator tip from touching the charging ring, another potential source of contamination.

In a typical determination, a microdroplet would be acquired in the trap under visual observation with helium neon laser illumination, 633 nm. The β-phycoerythrin has little absorption at this wavelength, so photolysis is minimal. After acquisition and centering in the trap, the observation port is closed, the HeNe laser blocked, the photon counter started, and the excitation laser switched on. The fluorescence signal decays in a few seconds to a background signal that is due to residual scattered light and luminescence from the trap and solvents within the droplet. After a time sufficient to observe the optical signal over many photochemical lifetimes of the analyte molecules, nominally 300 s, the droplet diameter is determined optically by reducing the laser intensity and measuring the displacement of the specular and refracted rays in the far field.[17] The accuracy of the diameter determinations is approximately 10%.

RESULTS AND DISCUSSION

The photocount rate versus time results for three different droplets are shown in Figure 3. The upper curve is for a droplet that is believed to contain one β-phycoerythrin molecule at the start of the determination. The β-phycoerythrin concentration in the solution was calculated to be 4.2×10^{-12} M in glycerol from the dilutions or 1.8×10^{-12} M

(17) Ashkin, A.; Dziedzic, J. M. *Appl. Opt.* 1981, *20*, 1803–1813.

Table I. Measurement Results for 24 Droplets Containing β-Phycoerythrin

sample no.	diam (μm)	volume (pL)	no. of analyte molecules	r
1	10	0.52	2	97
2	8	0.27	0	14
3	10	0.52	0	27
4	10	0.52	0	0
5	9	0.38	0	29
6	11	0.70	0	17
7	9	0.38	1	39
8	10	0.52	1	57
9	9	0.38	0	28
10	8	0.27	0	15
11	9	0.38	0	15
12	10	0.52	1	52
13	8	0.27	0	19
14	10	0.52	0	1
15	10	0.52	0	11
16	10	0.52	0	7
17	10	0.52	3	140
18	11	0.70	1	55
19	10	0.52	1	54
20	11	0.70	1	39
21	10	0.52	0	18
22	11	0.70	0	11
23	6	0.11	0	1
24	9	0.38	1	42

from the statistical appearance of analyte molecules in the droplets. The fluorescence is seen to decay to background with a time constant of about 3.5 s. The middle curve is for a droplet of blank solvent. The lower curve is for a droplet from the same solution as the upper curve, showing the absence of analyte molecules.

The results of 24 separate determinations on two different solutions of the same nominal concentration of β-phycoerythrin are presented in Table I. A total of 12 analyte molecules was observed in the 24 droplets with an average volume of 0.455×10^{-12} L, giving an experimental concentration of 1.8 pM. This result is considered to be in good agreement with the calculated concentration in glycerol of 4.2 pM in view of the uncertainties. The appearance frequencies of analyte molecules also closely correspond to those calculated from Poisson statistics. For droplets with 9.5-μm diameter and 1.8 pM concentration, the probabilities of 0, 1, 2, and 3 molecules being present are 0.619, 0.297, 0.071, and 0.011, respectively. The experimental probabilities, neglecting variations in droplet diameter, are 0.625, 0.292, 0.041, and 0.041, respectively. The close agreement of the larger values gives additional support to our interpretation of the results. The values of r presented in Table I are the peak values of the photocounts s^{-1} during the first 3 s after the excitation was switched on. This procedure was chosen because the photon counter was not synchronous with the excitation switching, and the fluorescence of the analyte molecules decayed rapidly in a few seconds, as seen in Figure 3.

The data for the 24 measurements in Table I are plotted in histogram form in Figure 4. Also shown are probability distribution functions for 0, 1, 2, and 3 molecules per droplet. When the maximum of 3 points is chosen, the distribution will no longer be Gaussian but biased toward values above the median. We have used probability distributions of the form

$$p_i(x) = 3[\int_{-\infty}^{x} \phi(x)\, dx]^2 \phi(x) \qquad (20)$$

where $\phi(x)$ is the normal distribution (Gaussian) in Figure 4. The areas of the four probability distributions have been made proportional to the relative probabilities of the four conditions from Poisson statistics. In calculating the positions

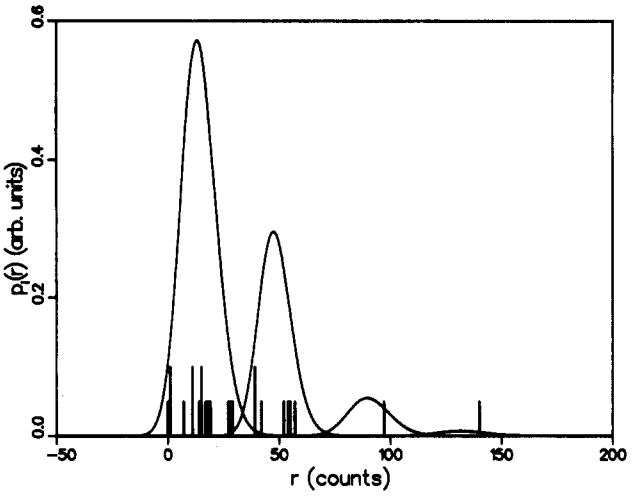

Figure 4. The experimental data for 24 droplets, together with calculated probability distribution. The vertical lines show the number of droplets, 0, 1, or 2, having r photocounts s^{-1}. The probability distributions from left to right correspond to 0, 1, 2, and 3 β-phycoerythrin molecules in a given droplet and are normalized to the Poisson probability calculated from the experimental concentration and sample volume.

and widths of the distribution curves, we used the experimental means and variances for the null and single molecule curves, corrected for the above distortion. The origins for 2 and 3 molecules were 2 and 3 times the single molecule origin. The variances for 2 and 3 molecules were taken to be the sum of the photocount values and the difference between the single molecule variance and the photocount value. We obtain for 0 molecules, $r = 9 \pm 9$ counts; 1 molecule, $r = 40 \pm 8$ counts, 2 molecules, $r = 80 \pm 10$ counts; 3 molecules, $r = 120 \pm 12$ counts. That the null value is nonzero may indicate that we have incorrectly identified some of the null droplets.

Seventeen droplets with a total volume of 6.1 pL that were deliberate blanks were measured over a 4-day period. Of these blanks, only one droplet showed a fluorescence signal that could be construed as due to a molecule of β-phycoerythrin. Two others showed an additional photoresponse above background that indicated some contamination but well below the integrated number of photocounts for an average β-phycoerythrin molecule. While the small number of samples makes the results quite uncertain, a rough estimate is that our sampling procedure may contribute a level of contamination equivalent to a 0.3 pM solution of β-phycoerythrin. This concentration might be considered to be an estimate of our present detection limit. Since it is unlikely that either the water or the glycerol contains β-phycoerythrin at this concentration, it is expected that the blank concentration can be further reduced if a droplet generation technique that minimizes samples handling can be developed.

The above results show that digital analysis as we have described it is feasible for certain analyte molecules. Experiments are not restricted to strongly fluorescing molecules since these can often be attached as fluorescence tags to nonfluorescing or weakly fluorescing molecules under study. The useful duration of a fluorescence determination is limited by the photochemical lifetime of the analyte molecule. Hence a better limit of detection can be achieved by digital measurements on small droplets than by a single, albeit more rapid, measurement on the bulk solution. The experiments described above required a substantial measurement time per sample. To take full advantage of digital detection methods, considerable improvement in measurement rate must be achieved. However, the only fundamental limitation is the fluorescence lifetime of the analyte molecule.

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