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A Statistical Analysis of Fluorescence Correlation Data

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Fluorescence correlation spectroscopy (FCS) is a relatively recent technique in which the diffusion coefficient of fluorescently labeled molecules can be determined. The change in diffusion behavior when these molecules interact with others can also be used to study interactions in solution. A new statistical method is proposed to analyze FCS measurements that cannot be evaluated with a classical autocorrelation function, which is normally used to analyze FCS data. It applies to binding studies where one of the interacting particles has a much brighter fluorescence intensity with respect to the other, which causes high fluorescence bursts whenever these molecules are detected. This biases the autocorrelation function, making it in most cases impossible to use this function as a fitting equation. Here, a statistical approach is used to quantify the amount of fluorescence found in bursts, thereby enabling to perform binding studies in cases where the fluorescence per molecule of both interacting species differs greatly. The method is demonstrated on a system of known composition, making it a promising tool for future FCS measurements.

KEY WORDS: Fluorescence correlation spectroscopy; statistics; fluorescein; interaction.

INTRODUCTION

Fluorescence correlation spectroscopy (FCS) is a relatively new experimental method in which diffusion coefficients of molecules can be determined [1,2; for reviews see Refs. 3 and 4]. A laser beam is focused into a solution of the molecules of interest, exciting only the particles that are in the focused beam at a certain point in time. In this way, fluorescence fluctuations are detected that reflect the diffusion of the molecules through the beam. When these ligands bind to bigger molecules that are themselves not excitable by the laser wavelength, fast and slow fluorescence fluctuations will occur that can be quantified in an autocorrelation analysis. The diffusion coefficients and fractions of both fast ligands and slow complexes can thus be determined and a binding study can be performed.

However, when the fluorescence quantum yield of the ligand increases strongly upon binding to its receptor or when several ligands interact with one molecule, the fluorescence emitted by a complex is much higher than that of free ligand, which causes the fraction of complexes to be overestimated. To a relatively small degree this can be corrected for (see Materials and Methods); in more extreme cases however, an autocorrelation analysis is no longer feasible (see Results).

We propose a statistical method to analyze measurements in which this problem occurs. When only free ligands are present in solution, the measured fluorescence intensities show a normal or Gaussian distribution, with the mean value determined by the mean number of molecules in the laser beam and their fluorescence quantum yield. When—in addition to these free molecules—complexes are present to which many ligands are bound, peaks of fluorescence are generated, which will cause the normal distribution of intensities to be too heavily weighted toward high fluorescence values. We establish

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a statistical test that can determine above which fluorescence value the measured intensities no longer contribute to the Gaussian distribution and are therefore regarded as complexes.

In biochemical systems this situation often occurs: binding of hormones to receptors on cells, binding of paclitaxel and microtubule associated proteins to microtubules, and interaction of intercalating dyes with DNA molecules are only a few examples. Therefore this analysis will be a valuable tool in many further FCS experiments.

MATERIALS AND METHODS

Chemicals

Fluorescein was purchased from Vel (Leuven, Belgium), 0.1-μm beads labeled with fluorescein were from Polysciences (Warrington, PA), KH_2PO_4 was from Merck (Darmstadt, Germany), and KCl was from BDH Laboratory Supplies (Poole, England). All chemicals were analytical grade.

Fluorescence Correlation Spectroscopy

In FCS, fluorescently labeled molecules are observed in the femtoliter excitation volume of a confocal microscope. Their average residence time in this open volume (diffusion time) can be assessed in an autocorrelation analysis. In this technique, use is made of the fact that fluorescence intensity values that are measured a short time interval τ after each other are correlated. This correlation is lost as τ increases. In the autocorrelation function $G(\tau)$, the fluorescence intensity at a certain time t is multiplied by the intensity a variable time interval τ later and this product is averaged for each interval. $G(\tau)$ is shown in a normalized form in Eq. (1) [1].

$$G(\tau) = \frac{\langle F(t)\rangle^2 + \langle \delta F(t)\, \delta F(t+\tau)\rangle}{\langle F(t)\rangle^2} \tag{1}$$

$\langle F(t)\rangle$ is the average fluorescence and δF the deviation from the average fluorescence at time t or a time interval τ later.

The function will show a decay when plotted against τ. It can be shown that the decay time equals the diffusion time (τ_d) of the studied molecules, which will be small for fast moving, thus small molecules [Eqs. (2) and (3)] [5].

$$G(\tau) = 1 + \frac{1}{N} f(\tau/\tau_d) \tag{2}$$

with

$$f(\tau/\tau_d) = \left[\frac{1}{1+\tau/\tau_d}\right]\left[\frac{1}{1+(\omega_1/\omega_2)^2\,\tau/\tau_d}\right]^{1/2} \tag{3}$$

ω_1 and ω_2 denote the half axes of the excitation volume perpendicular to and along the laser beam, respectively, and N the average number of fluorescent particles in the excitation volume. The mathematical derivation of Eqs. (2) and (3) from Eq. (1) is described by Elson and Magde [6]. The diffusion time τ_d is directly related to the diffusion coefficient D according to Eq. (4).

$$\tau_d = \frac{\omega_1^2}{4D} \tag{4}$$

When the labeled molecules bind to other, nonlabeled particles (at a 1-to-1 ratio), both the free ligands and the formed complexes show a characteristic diffusion time. In the autocorrelation function, the amplitude fractions of the fast and slow decay then reflect the fractions of free and bound ligand present [Eq. (5)] [5].

$$G(\tau) = 1 + \frac{1}{N_{app}}[(1-y)\cdot f(\tau/\tau_{free}) + y\cdot f(\tau/\tau_{bound})] \tag{5}$$

$1-y$ and y are the free and bound fraction of fluorescent ligand, respectively, and τ_{free} and τ_{bound} are the diffusion times of free and bound ligand, respectively.

The amplitude of this function is determined by the inverse of the apparent total particle number (N_{app}), which coincides with the actual particle number only when the free and bound ligands show the same fluorescence signal per molecule (fpm). If not, then the amplitude is primarily determined by the molecules with the highest fpm [Eq. (6)] [6]:

$$G(0) = 1 + \frac{\Sigma N_i \cdot \mathrm{fpm}_i^2}{(\Sigma N_i \cdot \mathrm{fpm}_i)^2} \tag{6}$$

N_i and fpm_i are the average number of molecules and the fluorescence per molecule, respectively, of each species i present.

In the case where two species are present, differences in fpm can be corrected for to a limited extent using the ratio of the fluorescence signals per particle (α) as a correction factor [Eqs. (7) and (8)] [5]:

$$G(\tau) = 1 + \frac{1}{N}\left[\frac{(1 - c) \cdot f(\tau/\tau_{free}) + c \cdot \alpha^2 \cdot f(\tau/\tau_{bound})}{[(1 - c) + c \cdot \alpha]^2}\right] \quad (7)$$

with

$$\alpha = \frac{\text{fpm}_{\text{bound ligand}}}{\text{fpm}_{\text{free ligand}}} \quad (8)$$

$1 - c$ and c are the actual fractions of free and bound ligand, respectively, taking into account their difference in fluorescence signal per molecule.

However, when several ligands bind to one receptor, the complexity of the system increases for two reasons. First, depending on the number of binding sites on one receptor and the concentration of ligand added to the system, the bound ligands will show a distribution over mono- and multiliganded receptors. Second, if the number of binding sites on one receptor—and so the fluorescence signal per complex—is quite high, as in the case of binding to DNA, protein polymers, or bacteria, the fraction of free ligand is no longer visible in the amplitude of the autocorrelation function, and a binding study using FCS is not possible.

Statistics

The problems in the FCS analysis of a system with multiple binding sites can be circumvented by simply quantifying the amount of fluorescence in the bursts created by the complexes and using this value as a measure for the amount of bound ligand. In this way, the advantages of a FCS setup (low amount of sample needed, no necessity for separation of free and bound ligand) are maintained, but the data are analyzed in a different way.

A repetition of the Kolmogorov–Smirnov goodness-of-fit test [7] is used to determine the amount of fluorescence signal caused by multiliganded complexes. The measured fluorescence intensities are ordered according to magnitude, and subsets of the data (gradually increasing in size and starting at the lowest values) are tested for normality. The Kolmogorov–Smirnov statistic measures the maximal deviation between the empirical distribution function of the test subset and the best-fitting normal distribution function. When this deviation is small, the subset is likely to be drawn from a normal distribution. When, on the contrary, it is large, the hypothesis that the data belong to a normal distribution becomes unlikely.

The result of the Kolmogorov–Smirnov test, the so-called p-value [7], reflects the probability that the data of the observed subset belong to a Gaussian distribution. More specifically, the p-value is the probability that, under the hypothesis that the data are drawn from a normal distribution, the Kolmogorov–Smirnov statistic is at least as large as the observed deviation. Thus, for normally distributed samples the p-value will be large, and for manifestly nonnormal samples it will be small.

When subsets with only the lower fluorescence values are tested, the distribution is truncated and the p-value is accordingly low (i.e., the test detects nonnormality). When larger values are gradually included into the test subset, a normal distribution around the mean becomes more likely and the p-value rises. When fluorescence peaks are also included into the subset, the distribution is biased toward high values and the p-value again drops to a low level. In this way, the upper fluorescence value of the largest subset with a p-value above a chosen significance level, in this case 0.05, can be considered as the limit above which the measured intensities are regarded as belonging to complexes.

Setup and Measurements

A FCS setup was used as described [8]. Measurements were performed at room temperature in a phosphate buffer [50 m*M* KH_2PO_4, with the ionic strength adjusted to 0.1 *M* with KCl (pH 7)] in sample volumes of 100 μl. Measuring time was 60 s (except for the measurements with only free fluorescein present: 40 s) and all samples were measured at least nine times. Where possible, an autocorrelation analysis was carried out. A self-written algorithm that implements the above statistical procedure in the S-Plus software package (Mathsoft, Inc.) was applied to all data. This algorithm is available upon request. Each FCS measurement yielded a set of 507–510 intensity values to be analyzed statistically. For measurements of 60 s this means that each value represents the intensity (kHz) measured during a time period of 0.12 s.

RESULTS

A multiple binding site system was simulated by mixing a nanomolar solution of a free fluorophore, fluorescein, with different quantities of a 15 p*M* stock solution of polystyrene beads (100-nm diameter), uniformly coated with the fluorophore. The final concentration of fluorescein was kept constant at 7 n*M*; final concentrations of beads in the samples were between 0.75 and 9 p*M*.

In a control experiment, a pure fluorescein sample was measured. The autocorrelation analysis yielded a typical one-component fit with a diffusion time of about 60 μs (Fig. 1A). Since no complexes were present in this

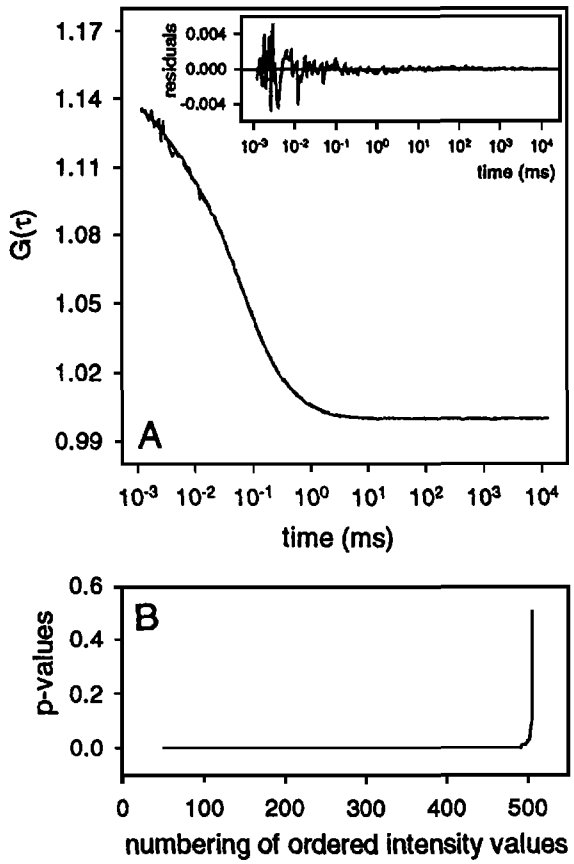

Fig. 1. Autocorrelation (A) and statistical (B) analysis of a sample containing fluorescein molecules (10 n*M*). (A) The one-component fit (dashed line) of the autocorrelation function (solid line) yields a diffusion time of 61.1 μs. The inset shows the residuals of the fit. (B) p-values resulting from the Kolmogorov–Smirnov tests on the sample. For technical reasons with respect to the software as implemented in S-Plus, all p-values equal to or above 0.05 are set to a value of 0.5. All data points are considered as part of the Gaussian intensity distribution.

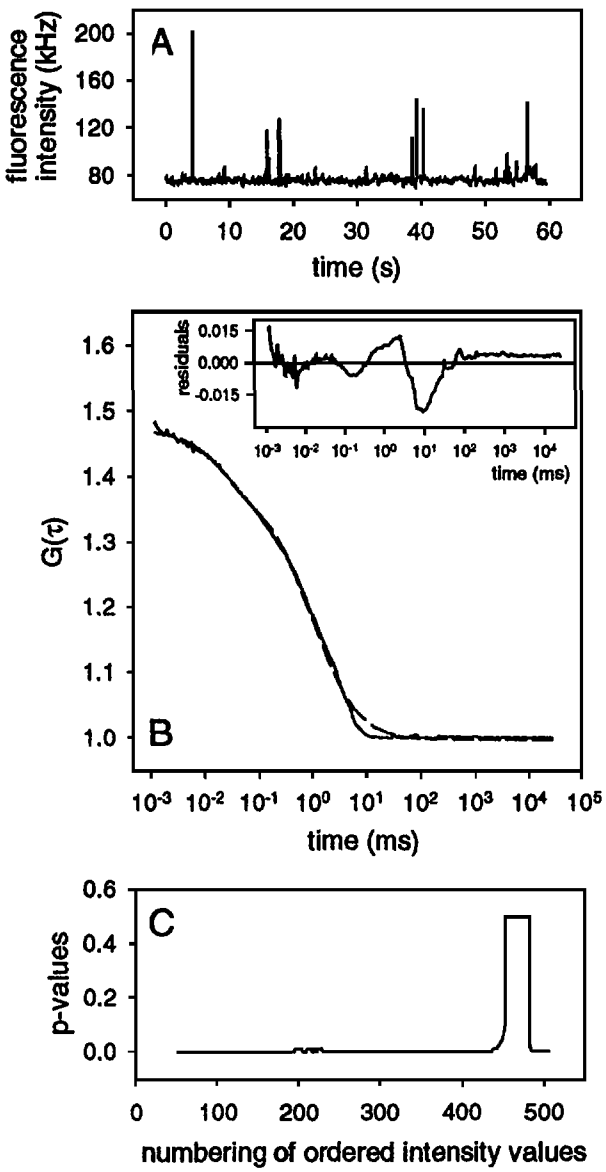

Fig. 2. Mixture of 0.75 p*M* beads with 7 n*M* fluorescein. (A) Fluorescence intensity data showing high fluorescence bursts when beads are detected. (B) Autocorrelation analysis of the sample. When the curve (solid line) is fitted to two components (dashed line), fixing the diffusion time of one component at the value of 61.1 μs found for free fluorescein, the fit results are 0% of this component and 100% of complexes with a diffusion time of 968.1 μs. The inset shows the residuals of the fit. (C) p-values resulting from the statistical analysis. For technical reasons with respect to the software as implemented in S-Plus, all p-values equal to or above 0.05 are set to a value of 0.5. Twenty-four of the 508 data points, corresponding to 4.7%, are attributed to beads.

solution, the statistical analysis should show no outliers and consider the complete set of fluorescence data as part of the normal distribution. As can be seen in Fig. 1B, this was indeed the case since the Kolmogorov–Smirnov goodness-of-fit test yielded a high p-value for the complete data set.

Subsequently, mixtures were made between the nanomolar fluorescein solution and a picomolar stock solution containing covalently coated beads. In Fig. 2, one FCS measurement of the mixture containing the smallest quantity of beads (0.75 p*M* beads in addition to 7 n*M* free fluorescein) is shown. The measured fluorescence intensities now clearly display peaks as the beads are passing through the excitation volume (Fig. 2A). Although the number of peaks does not seem extremely high, the autocorrelation function fits only to one component with a diffusion time of about 1 ms (Fig. 2B), meaning that the correlation analysis can no longer discriminate between free and bound fluorophores, even though the number of beads observed is not high. According to the

statistical analysis, the 484 lowest values of the 508 ordered intensities contribute to a Gaussian distribution; consequently the 24 highest values or 4.7% of the measured fluorescence intensities are found in bursts (Fig. 2C). This coincides with all values measured above 85.3 kHz. When the percentage of observed intensities in peaks is plotted against the concentration of beads present (Fig. 3), it is evident that the statistical results change linearly with the amount of beads in the sample. They can therefore be used as a probe for the unknown number of complexes in a binding study.

However, in an actual chemical binding one is interested in the amount of ligand bound, not merely in the number of complexes present. For a system in which the ligands are uniformly distributed over the receptors, as in the case of fluorescein coated beads, this does not complicate the data analysis. In a noncovalent binding to a receptor bearing multiple binding sites, on the contrary, ligands will show a nonuniform distribution over the receptors. This means that, in addition to the number of peaks, also their height has to be considered. For example, a complex bearing 10 ligands accounts for twice as much bound ligand as a complex with 5 ligands, but in the statistical analysis they can both give rise to the same number of outliers, but with a different intensity. In these cases, a more correct statistical approach therefore would be to sum the fluorescence intensities measured above the statistically determined limit and use this integral as a measure for the amount of bound ligand—instead of the amount of complexes—in the examined sample. In this approach, the results from different samples can be compared with each other provided the measurement time is the same.

It was examined whether this method gave a correct result when applied to the fluorescein–bead mixtures. A mean value was calculated of all intensities above the statistical limit, from which the mean of all data under this limit was subtracted. The result was then multiplied with the number of observations found in peaks. The sum of fluorescence signals attributed to beads was in this way corrected for the signal of free fluorophores present. Indeed, the linear behavior was also observed in this analysis (Fig. 4).

To confirm the validity of this method, it was applied to a series of experiments in which the concentration of free fluorescein was also altered in addition to the changes in the concentration of beads. Since this is normally the case in actual binding studies, it is a good demonstration of the capabilities of this technique. Indeed, the total fluorescence in peaks, corrected for free fluorophore, showed a linear behavior as a function of the concentration of beads (Fig. 5A). On the contrary, in an analysis in which we did not correct for the free fluorophores present, but simply made the sum of all intensity values found in peaks, the influence of an increasing background of free fluorescein with rising concentrations of beads was visible (Fig. 5B).

DISCUSSION

It was demonstrated that the proposed statistical analysis is an excellent method for determining the

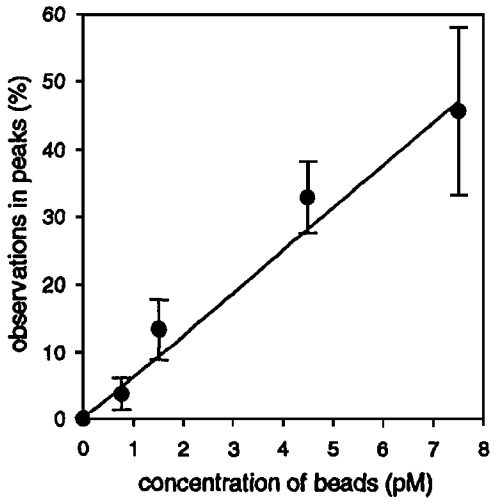

Fig. 3. Number of fluorescence intensities found in peaks, expressed as a percentage of the total amount of data points for each measurement, for different concentrations of beads in the sample. The fluorescein concentration was 7 n*M* in each experiment, concentrations of beads varied between 0.75 and 7.5 p*M*.

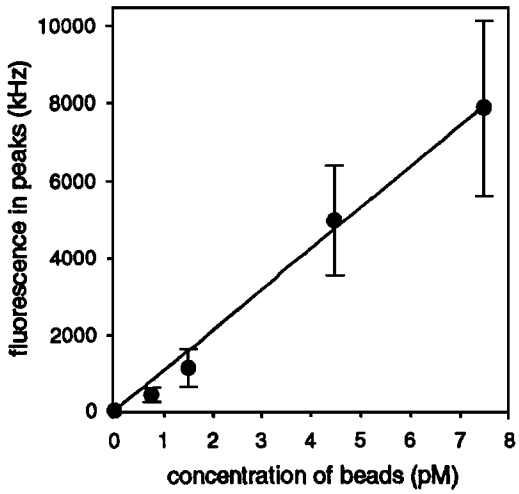

Fig. 4. Sum of the fluorescence found in peaks for the experiment shown in Fig. 3, after correction for the amount of free fluorescein in the sample.

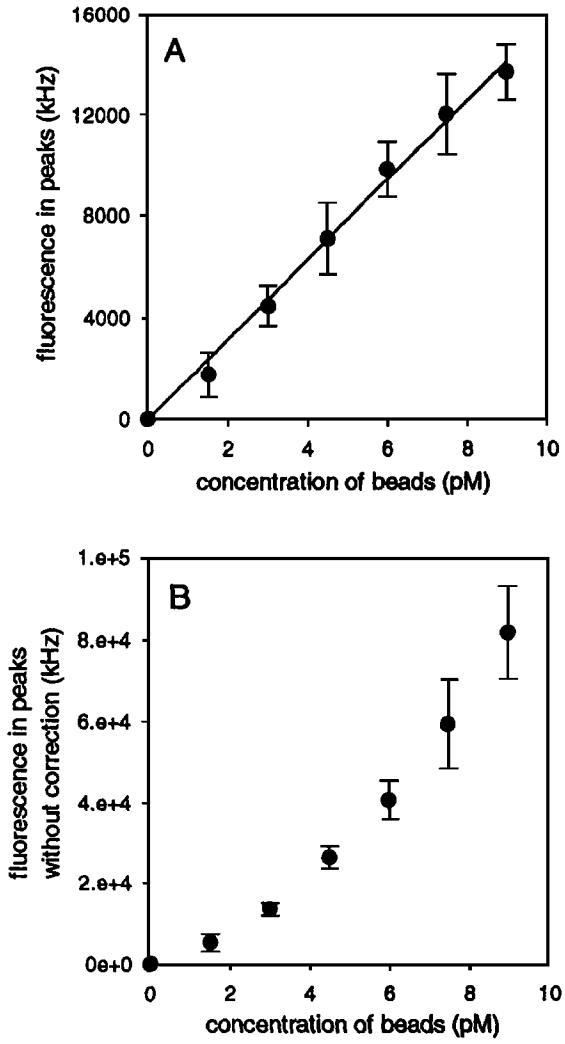

Fig. 5. Statistical analysis of a series of samples with both increasing fluorescein concentration (between 2.5 and 25 n*M*) and bead concentration (between 1.5 and 9 p*M*). (A) Total fluorescence found in peaks, corrected for the amount of free fluorophores present, plotted against the concentration of beads. (B) Sum of the fluorescence found in peaks, without any correction; as a function of the concentration of beads in the sample.

amount of bound ligand when analyzing interactions in which the fluorescence signals per molecule of the free ligands and of the complexes differ greatly. From the fluorescein–bead measurements, some conclusions can be made about how to process the fluorescence data for different systems.

In the case of a uniform distribution of ligands over the receptors, the number of observations in peaks can be used as a relative value for the amount of complexes present, as shown in the presented experiments.

In most biochemical systems, however, ligands show a nonuniform distribution over the receptors, and also the height of the peaks has to be accounted for. In these cases, the integrated fluorescence in the peaks, corrected for the presence of free fluorophores, is proportional to the quantity of bound ligand in the sample. Even in the case of a uniform distribution of ligands over receptors, this analysis can be of use since it takes into account the fact that two or more complexes can pass through the excitation volume during the same measurement time interval of 0.12 s, giving rise to a fluorescence burst with a high intensity.

The way to use the statistical results in an actual binding study is of course case dependent but can be demonstrated by means of the example illustrated in Fig. 5. An experiment in which one adds increasing concentrations of free fluorescent ligand to constant quantities of receptors carrying multiple binding sites can indeed result in a series of samples with an increasing occurrence both of peaks and of free ligand fluorescence. From such a series of measurements the association equilibrium constant can be deduced in the following manner: the binding of a ligand L to one binding site of the receptor R can be represented as in Eq. (9).

$$L + R \leftrightarrows LR \tag{9}$$

with the association equilibrium constant K_{ass} [Eq. (10)]:

$$K_{ass} = \frac{[LR]}{[L][R]} \tag{10}$$

[R], [LR], and [L] are the concentrations of free binding sites, saturated binding sites, and free ligand, respectively, at equilibrium.

From the expression for K_{ass}, the equation for a saturation curve is readily deduced [Eq. (11)]:

$$\Phi_{LR} = \frac{[LR]}{[R_{tot}]} = \frac{K_{ass}[L]}{1 + K_{ass}[L]} \tag{11}$$

Φ_{LR} is the degree of saturation of the binding sites and $[R_{tot}]$ the total concentration of binding sites or [R] + [LR]. If $[R_{tot}]$ is low in comparison to the total ligand concentration, [L] can be considered equal to the initially added total ligand concentration $[L]_{in}$, and K_{ass} can be determined when measuring Φ_{LR} in function of $[L]_{in}$.

This means that any measure of the concentration of bound ligand [LR], like the total fluorescence found in peaks, can be used to calculate K_{ass} with only a slight adjustment of Eq. (11) [Eq. (12)]:

$$X_{LR} = X_{max}\frac{K_{ass}[L]}{1 + K_{ass}[L]} \tag{12}$$

X_{LR} is the signal that expresses the quantity of bound ligand in the sample, and X_{max} is the maximum signal at high ligand concentrations.

This equation can be applied successfully, provided that the concentration of free ligand can be increased enough to reach saturation, i.e., the affinity of ligand for receptor has to be sufficiently high. In this example, the concentration of the fluorescently labeled ligand is varied, so one is limited to the nanomolar concentration range. However, it is equally possible to alter the concentration of the nonlabeled receptor, which can be varied over a much broader concentration range, thereby enabling to study interactions of lower affinity. The saturation analysis is in these conditions applied in an analogous way.

A possible error that can be made in this method is the fact that monoliganded receptors are disregarded in the statistical analysis. However, these species contribute significantly only to the first part of a binding curve. In a complete binding study over a sufficiently large concentration range of ligand, this deviation becomes negligible.

A saturation analysis is equally feasible when fluorescence bursts originate from an increase in the fluorescence signal per molecule when a ligand is bound to its receptor (with one or several binding sites), so it is generally applicable.

The proposed statistical method offers the possibility to perform binding studies in situations where an autocorrelation analysis does not permit an accurate discrimination between free and bound ligand. However, autocorrelation can still be applied in the measurements where no or very few fluorescence peaks are observed (see Figs. 1 and 2, respectively): even though the fitted fraction of bound ligand obtained from the measurement in Fig. 2 is seriously overestimated, the diffusion times of free ligands and complexes can eventually be assessed. In this way, complementary information on the system under study can be obtained from both autocorrelation analysis and the statistical method.

A related approach has been successfully applied by Pitschke *et al.* [9] in the analysis of the formation of amyloid β-protein aggregates in the cerebrospinal fluid of Alzheimer's patients. The frequency of peaks resulting from the aggregates was studied, fluorescence fluctuations were regarded as peaks when they were higher than three times the average fluctuation of the fluorescence intensity.

NOTE ADDED IN PROOF

While this paper was in press, an article has been published by Chen *et al.* [10], in which a home-made experimental setup equipped with a fast data acquisition card is used to perform a photon counting histogram analysis, that further extends the possibilities of peak analysis in FCS. Nevertheless, the more pragmatic method presented here can be a practical tool to study biochemical interactions in the experimental conditions mentioned in the present paper.

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