Local Stoichiometries Determined by Counting Individual Molecules

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The capability of optical single-molecule microscopy for quantitative determination of local stoichiometries is demonstrated. Biotin–streptavidin embedded in a phospholipid membrane was chosen as a model system. At a local resolution of 30 nm and a time resolution of 35 ms, it was possible to determine local stoichiometries between one and four occurring in this system. The “stoichiometric resolution” of the method is analyzed by statistical means. From this a quantitative criterion is derived for the reliability in assigning a defined number of fluorophores to each individual fluorescence peak observed. It is shown that only a few (n ≈ 10) observations are sufficient for reliable assignments.

Observations on the level of individual molecules make possible the ultimate goal in analytical chemistry, “digital analyte quantitation.” Most recent developments have extended these pioneering methods to observations of dynamic behavior with the ability for localization of individual fluorescent molecules to within 30 nm. These extended developments are particularly designed for in vivo studies of biological specimens relying on state-of-the-art fluorescence microscopy. By use of fluorescence-labeled ligands such as antibodies or drugs, direct imaging of individual molecules in physiological environments is now possible. It was shown that the photophysics and mobility of individual molecules labeled in a phospholipid membrane and individual ATP turnovers by single myosin molecules can be analyzed.

Optical methods to determine the stoichiometry of molecular associates that are laterally not resolved are of increasing interest in bioscience and analytical chemistry. A technique for measuring local stoichiometries of associates beyond the diffraction limit, denoted as superresolution, or microscopy in high dimensions, should be a powerful tool to study co-association of cellular components. From such technical advances one expects to gain new insights into the molecular organization and recognition underlying biological function. We have proposed that such superresolution pursued on the single-molecule level should be feasible. The strategy is to compare the local fluorescence intensity with the unitary fluorescence intensity, the fluorescence intensity from a single fluorophore. This should permit estimation of the number of fluorophores contributing to the total fluorescence signal up to a limiting number set by the “stoichiometric resolution” of the method. Such strategy has been used earlier, not at the single-fluorophore level but by employing multiple fluorescence labeling. It was possible to determine clustering of low-density lipoproteins on cell surfaces and DNA sizes and molecular weights. However, the main advantage of single-fluorophore counting with respect to the determination of local stoichiometries is the a priori knowledge of the unitary fluorescence intensity. In addition, possible interference of labeling with the biological function is reduced to a minimum.

In the current paper, we investigate the potential for single-molecule imaging in determining the stoichiometry for the model system of biotin–streptavidin. Streptavidin is a bacterial protein that consists of four identical subunits, each binding one biotin molecule with extremely high affinity (binding constant Kd = 10 fM). Experiments were performed on biotin–streptavidin complexes linked to phospholipid membranes with a local resolution of ~30 nm. By use of a mixture of fluorescence-labeled biotin and nonfluorescent biotin this system appeared ideally suited for the present purpose since it provides a reliable basis for realization of stoichiometries between 1 and 4. In addition to stoichiometric assignment, the time resolution of milliseconds allowed determination of diffusion constants of biotin–streptavidin complexes in fluid and gellike phospholipid membranes and to study association phenomena.

EXPERIMENTAL SECTION

Reagents. Supported phospholipid membranes of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Sigma) were deposited from a monolayer trough (M ayer Fein technik, Göttingen, Germany) on glass substrates using the Langmuir–Blodgett technique as described in ref 9. The coated substrate, mounted onto a quartz cuvette (10 μm, QS-106, Hellma), was continuously flushed with PBS buffer (150 mM NaCl, 10 mM NaHPO4, pH 7.4). The external layer of this membrane contained small amounts (10−6 mol/mol of lipid) of biotin–X-DMPE (1,2-dimyristoyl-sn-
glycero-3-phosphoethanolamine derivate, B-1616, Molecular Probes) equivalent to a surface density of ~0.1 μm−2.7 The biotinylated membrane was incubated with 50 nM streptavidin (Sigma) for ~3 min, flushed with buffer for ~5 min, and subsequently incubated with 50 nM of a long-chain biotin−dye conjugate (biotin−PEG-TMR) for longer than 10 min. The latter molecule has been synthesized in our group15 by derivatization of O,O′-bis-(2-aminopropyl)poly(ethylene glycol) 1900 (14529, Fluka) with the N-hydroxysuccinimidyl esters of biotin (S-1513, Molecular Probes) and of 5-carboxytetramethylrhodamine (C-2211, Molecular Probes).16 The biotin−PEG derivatives were not further purified, yielding a photometrically determined mixture of TM R-labeled and unlabeled biotin−PEG in a ratio of 3:1. Usage of the long-chain biotin−dye conjugate was essential for the current investigation. The fluorescence quantum yield of biotin−PEG-TMR decreased to 70 ± 10% upon binding to streptavidin,16 whereas it decreased by a factor of 10 for a commercially available short-chain biotin−dye conjugate (biotin−XX-TMR). No binding of the long-chain biotin−PEG-TMR to the phospholipid membrane was observed when the preincubation step with streptavidin was omitted.

Apparatus and Data Analysis. The apparatus, data acquisition, and automatic data analysis system used were as previously described in detail.5 In brief, samples were observed while illuminated for 5 ms by 25 ± 6 kW/cm2 of 528 nm circular polarized light from an Ar+ laser (C306, Coherent) using a ×100 objective (PlanNeofluar, NA = 1.3, Zeiss) in an epifluorescence microscope (Axiovert 135TV, Zeiss). The fluorescence was effectively separated from Rayleigh and Raman-shifted light by appropriate filter combinations (515DRLPEXT02 dichroic, 570DF70 block, Omega, OG550 low-pass, Schott). Data were obtained at a rate of 29 images/s using a modified liquid nitrogen-cooled slow-scan CCD—camera system (AT200, Photometrix, equipped with a TK512CB-chip, Tektronix) and stored on a PC. An automatic analysis program determined the position of each fluorescence complex to within ∼30 nm and its fluorescence power to ∼20% accuracy by fitting the fluorescence intensity profile to a two-dimensional Gaussian surface. The uncertainties are fully accounted for by the signal-to-noise ratio of the system as shown by simulations.7 From the shifts in position for subsequent images, the two-dimensional trajectory for each complex within the phospholipid membrane was reconstructed. Averaging multiple trajectories allowed a mean-square-displacement analysis for determination of the lateral diffusion constant.

Criteria for Stoichiometry Assignments. Reports on the observation of individual fluorophores in phospholipid membranes7,9 provide an a priori knowledge for the fluorescence intensity of an individual fluorophore embedded in a biomembrane. For the excitation intensity of 25 kW/cm2 and illumination time of 5 ms, as used throughout this study, the single-fluorophore intensity is characterized by a distribution with a mean of I1 = 170 counts and a width of σ1 = 67 counts5 (see Figure 1). For I1 and σ1 given, the fluorescence intensity for N-independent, co-localized fluorophores is I = N1 ± N1/2σ1. This provides a frame of reference for assigning a particular N value, i.e., stoichiometry, to each intensity peak observed. The assignment itself is the first objective in this subsection. Subsequently, a measure is proposed for the reliability of such assignments which is shown to be related to the stoichiometric resolution, Nmax, as introduced in ref 7. Nmax is defined by the N value, or stoichiometry, for which the uncertainty N1/2σ1 of determining I1 equals I1, the mean fluorescence intensity of single fluorophores:

\[ N_{\text{max}} = \left( \frac{i_1}{\sigma_1} \right)^2 \]  

(1)

For the experimental conditions used throughout this study Nmax = (170/67)2 = 6.4.

An isolated image of co-localized fluorescent dye molecules will be similar to that of a single dye but with higher fluorescence intensity. In general, for any given intensity distribution of individual fluorophores, I1, the corresponding intensity distribution of N co-localized fluorophores, I N (l), can be calculated recursively as a series of convolution integrals:

\[ I_N(l) = \int_{-l}^{l} I_1(l') \rho_N(l-l') dl' \]  

(2)

In eq 2 the intensity values of the fluorescent molecules are assumed to be independent from each other. This requirement was fulfilled by introducing a long spacer molecule15 between the biotin and the fluorophore as shown in fluorescence yield studies.16

\[ \rho_N(l) = \int_{-l}^{l} \rho_{N-1}(l-l') \rho_{N-l}(1-l') \]  

(3)
Figure 1a shows the calculated intensity distributions predicted for fluorescence peaks containing two to nine molecules. $\rho_1$ was experimentally determined in ref 9. The distribution was constructed in the form of a probability density function\(^9\) from experimental intensity profiles of 342 individual fluorophores. In addition, the intensity distribution of a sample containing no fluorescence molecules, $\rho_0$, is included in the figure. All distributions can be approximated by Gaussians, in particular for higher $N$ values. These distributions provide the basis for further classification of individual fluorescence peaks including the local stoichiometry, $N$, the probability, and the reliability of this particular assignment.

The probability for assigning a peak intensity, $I \pm \Delta I$, to a certain number of fluorescence molecules or stoichiometry, $N$, is given by\(^7\)

$$p(N|I \pm \Delta I) = \frac{p(I \pm \Delta I|N)}{\sum_n p(I \pm \Delta I|n)}$$  \hspace{1cm} (3)

with the conditional probability density for finding the intensity within $I \pm \Delta I$ for a given stoichiometry $N$:

$$p(I \pm \Delta I|N) = \frac{1}{2\pi \Delta I^2} \int dI' \rho_N(I') \exp \left(-\frac{(I'-I)^2}{2\Delta I^2}\right)$$  \hspace{1cm} (4)

Values for $p(N|I \pm \Delta I)$ are plotted in Figure 1b for $\Delta I = 35$ counts, which was a typical value in our experiments. The $N$ value for which $p(N|I \pm \Delta I)$ is maximal is the most appropriate assignment of a number $N$ of co-localized dye molecules to an individually fluorescence peak with intensity $I \pm \Delta I$ (Bayes criterion\(^8\)). The distributions shown in Figure 1b yield $N = 0$ for $I < 48$ counts, $N = 1$ between 48 and 240 counts, $N = 2$ between 241 and 412 counts, $N = 3$ between 413 and 584 counts, $N = 4$ between 585 and 756 counts, and $N = 5$ between 757 and 924 counts. These ranges are indicated on top of Figure 1b.

In this way the intensity scale is divided into ranges for assigning most probable $N$ values to observed peaks. However, the probability value is an inappropriate measure for the quality or reliability of assigning an intensity to one particular $N$ value. For fluorescence intensities falling at either end of the ranges, the probabilities of the two adjacent $N$ values become close so that it becomes impossible to make one particular assignment. One way to construct an appropriate measure of this is a reliability function, $r(N|I \pm \Delta I)$ of the form

$$r(N|I \pm \Delta I) = \frac{p(N|I \pm \Delta I) - p(N \pm 1|I \pm \Delta I)}{p(N|I \pm \Delta I) + p(N \pm 1|I \pm \Delta I)}$$  \hspace{1cm} (5)

with $p(N \pm 1|I \pm \Delta I)$ denoting the maximum of $p(N - 1|I \pm \Delta I)$ and $p(N + 1|I \pm \Delta I)$. The reliability function is defined such that it approaches zero for $I$ values with equal probabilities for two stoichiometries. The values of $r(N|I \pm \Delta I)$ are plotted in Figure 1c. We define here an assignment that fulfills $r > 0.25$ as reliable (dotted line). The rationale for this criterion is seen from an analytical approximation of eq 5:

$$r(N|l) = \frac{1 - G(N|I)}{1 + G(N|I)}$$

with

$$G(N|I) = \exp \left(-\frac{N_{\text{max}}}{2N} \left(1 - \frac{2|l|}{I_1}\right)^2\right); \quad \delta l(N) = |l - N_{11}|$$  \hspace{1cm} (6)

It is readily obtained by neglecting the uncertainty $\delta l$ in the determination of $l$, giving $p(l \pm \delta I|N) = \rho_N(l)$ and, further, by assuming $\rho_N$ and $\rho_{N+1}$ to be Gaussians with identical variances. The criterion introduced for reliable assignment of individual fluorophores, $r > 0.25$, is then obtained from $r(N_{\text{max}}|N_{\text{max}}) = (1 - e^{-3})/(1 + e^{-3}) = 0.25$. This yields a range of intensities for each $N$, $\delta I(N)$, within which an assignment is reliable:

$$\delta l(N) = \left(q - \frac{N_{11}}{N_{\text{max}}}\right)$$  \hspace{1cm} (7)

This criterion includes that assignments of $N$ co-localized fluorophores are limited to $N < N_{\text{max}}$, the stoichiometric resolution. For applications we note that the criterion $r > 0.25$ is independent of experimental data, that the values of $r$ are determined using the a priori values $l_1$ and $\alpha_1$, and that the approximate relations in eqs 6 and 7 are sufficient accurate for most practical purposes.

In this way each fluorescence peak $I \pm \Delta I$ is classified by a stoichiometry $N$ and a probability $p(N|I \pm \Delta I)$, both determined from eq 3, and a reliability $r(N|I \pm \Delta I)$ calculated from eq 5 or 6 that is to be compared to the reliability limit of 0.25.

**RESULTS AND DISCUSSION**

Figure 2a shows a typical fluorescence image of a phospholipid (POPC) membrane containing ~0.1 streptavidin molecule/µm² after incubation with 50 nM of the fluorescence-labeled biotin. The isolated fluorescence peaks of apparently different intensities reflect defined numbers of fluorescent biotin molecules bound to individual streptavidin molecules.

For a detailed analysis, the peaks were approached by two-dimensional Gaussian surfaces from which their intensities, $I$, and respective confidence intervals, $\Delta I$, were determined.\(^9\) The corresponding stoichiometry value, $N$, was subsequently determined from the maximal probability calculated from eq 3. Results for the data in Figure 2a are shown in Figure 2b with values of $N$ between 1 and 4 indicated by the heights of the cylindrical symbols. For each data point two further numbers, the probability $p(N|I \pm \Delta I)$ and the reliability $r(N|I \pm \Delta I)$ are given, as calculated from eqs 3 and 5, respectively.

The $r$ values for the two peaks for $N = 1$ are both above 0.25, so that assignment of these fluorescence peaks to single fluorophores appears reliable. This is expected from the extended reliability range of $I$ seen for $N = 1$ in Figure 1c and from the predicted mean reliability of $(r(I, 1 \pm \Delta I)) = 0.65$. This changes for the four fluorescence peaks assigned to $N = 2$. Only one of them meets the reliability criterion $r > 0.25$; the other three values of $r$ are below the reliability threshold. This is consistent with the predicted mean value of $(r(2, 1 \pm \Delta I)) = 0.23$. Apparently, for the experimental conditions given, the assignment of stoichiometries of $N = 2$ on the level of individual data points is at the limit of reliability. In this case, one must consider small ensembles for reliable evidence of co-localization. For the data shown in Figure 2, the average intensity of the four fluorescence peaks assigned to $N = 2$, $I = 272 \pm 11$ counts, meets the reliability criterion $(r(2|l) = 0.30$ and $p(2|l) = 0.59)$. This was similar for the fluorescence peak assigned to $N = 4$ in Figure 2. When combined with 11 further peaks of $N = 4$, the mean intensity $I = 677 \pm 18$ counts fulfilled the reliability criterion $(r(4|l) = 0.34$ and $p(4|l) = 0.51)$. This shows that the reliability of assigning a

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stoichiometry value, N, to a single fluorescence peak depends on the value of N. For N = 1, almost each observation is reliably assigned. For larger values of N a small ensemble (n ≈ 10) of observations appears to be sufficient (for the i1 and σ1 values given) to fulfill the reliability criterion, which is taken as evidence for the existence of that particular stoichiometry.

Such assignments, made on the basis of one to a few data for each N value could be confirmed by analysis of large ensembles. The overall intensity probability density function, p, is shown in Figure 3. p was constructed from 108 fluorescence peaks by taking into account the individual intensity and confidence interval. The assignment of N values to all data yield stoichiometries between 1 and 4. The mean intensity, iN, for each stoichiometry, N = 1, 2, 3, and 4, is plotted in the inset of Figure 3 (open circles). iN is given by the mean of all peaks that had been assigned to the same N value. As expected, iN increases linearly with N yielding i1 = 162 ± 3 counts. This value is in excellent agreement with i1 = 170 counts, which has been used in this study as the a priori value for stoichiometric assignment of individual fluorescence peaks. This demonstrates that by careful choice of an independent "fluorescence standard" the a priori value of i1, can be closely estimated.

Alternatively, a value for i1 may be estimated from the overall probability density function without making use of prior stoichiometry assignments. For this, the positions of the four maxima were estimated by fit of four Gaussians and included in the inset of Figure 3 for comparison (solid circles). The mean interval is 142 ± 8 counts, which is smaller than i1 = 162 ± 3 counts. The difference is explained by the systematic deviation of the Gaussians from the experimental distribution functions, which decrease

Figure 2. (a) Fluorescence images of a 9.6 × 9.6 µm² area of a POPC membrane. The membrane contained fluorescence-labeled and unlabeled biotin molecules bound to streptavidin at very low surface density. (b) The number of cylinders indicate the number of fluorophores, N, assigned to the fluorescence peaks in (a). The two numbers given below each symbol (ρ, τ) are the probability and the reliability of these assignments calculated from eqs 3 and 5, respectively. Fluorescence intensities (from left to right): N = 1 (219, 155 counts); N = 2 (262, 259, 305, 263 counts); N = 4 (637 counts).
with increasing N. In particular, the distribution for N = 1 is considerably asymmetric (see Figure 1a), which mainly accounts for the deviations from the fitted Gaussian profile at low intensity.

The relative occurrences of N values and the mean, N̄, were determined from areas of Gaussian fits. Analysis of seven samples with 660 peaks in total for fluid and gel-like membranes yielded the data listed in Table 1. It should be mentioned that identical peaks in both fluid and gel-like membranes yielded the same frequency although their mobility in the two types of membranes was significantly different as is evident from the lateral diffusion constants (see Table 1). We carried out a binomial analysis of biotin binding to streptavidin, taking into account the ratio of fluorescence labeled to unlabeled biotin of 3:1. This yielded occurrences of streptavidin monomers and dimers. The small deviations can be explained by photobleaching, the process of light-induced destruction of a fluorophore. In some of the trajectories, a decremental loss of the fluorescence intensity of individual peaks was observed, which we interpret by stepwise photobleaching of individual fluorophores in the biotin–streptavidin complex.

**CONCLUSION**

We have determined stoichiometries of individual ligand–receptor complexes in a digital manner, making use of the quantal nature of the fluorescence from individual ligands on a receptor protein. Such detailed intensity analysis combined with the spatial and temporal possibilities of state-of-the-art video-enhanced fluorescence microscopy makes single-molecule studies an attractive opportunity to study local stoichiometries and aggregation in complex systems such as biomembranes.

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**Table 1. Local Stoichiometries, N, of Fluorescence-Labeled Biotin Bound to Streptavidin on Two Different Phospholipid Membranes**

<table>
<thead>
<tr>
<th>N</th>
<th>fluid membrane (POPC)</th>
<th>gel-like membrane (DMPC)</th>
<th>predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32 ± 9</td>
<td>33 ± 5</td>
<td>29</td>
</tr>
<tr>
<td>2</td>
<td>40 ± 6</td>
<td>43 ± 6</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>14 ± 5</td>
<td>20 ± 3</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>14 ± 8</td>
<td>4 ± 3</td>
<td>11</td>
</tr>
<tr>
<td>N̄</td>
<td>2.1 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The numbers refer to analysis by Gaussian fits of the intensity distributions obtained for 426 (POPC–membrane) and 234 (DM PC–membrane) biotin–streptavidin complexes, respectively. The lateral diffusion constants, Dlat, were determined on the basis of trajectories of individual streptavidin molecules. Predicted values were calculated by assuming independent binding of biotin molecules to streptavidin and taking into account the 3:1 ratio of the fluorescence-labeled to unlabeled biotin used. The streptavidin monomers were assumed to occur twice as often as streptavidin dimers. Dlat = (2.7 ± 0.5 × 10⁻⁸ cm/s) × (0.16 ± 0.05) × 10⁻⁸ cm²/s.


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