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Determining Antibody Stoichiometry using Time-Integrated Fluorescence Cumulant Analysis

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Abstract

We applied fluorescence fluctuation spectroscopy to resolve binding heterogeneity of fluorescently labeled ligand derived from brain natriuretic peptide (BNP), a widely used diagnostic marker of heart failure, to a corresponding monoclonal antibody. This system includes three species: (1) free ligand molecules, (2) antibody with a single site occupied and (3) antibody with both sites occupied. The method we used, time-integrated fluorescence cumulant analysis (TIFCA), utilizes cumulants of fluorescence fluctuations to resolve subpopulations of multiple fluorescent species freely diffusing in a solution. The values of the cumulants depend on the concentration, molecular brightness and diffusion time of the fluorescent molecules. The number of molecules in each species reflects the antibody affinity. We apply TIFCA to successfully establish the stoichiometry of the system, estimate affinity, and identify the presence of an inactive fraction of antigen in a single titration experiment.

Keywords

Fluorescence Spectroscopy; Photon Statistics; Antibody; Stoichiometry; Ligand Binding

1. Introduction

Studying protein binding equilibria is a classical topic of physical chemistry¹⁻³. Such studies are based on thermodynamic principles and traditionally have been performed by analyzing protein ensembles without direct measurement of binding heterogeneity⁴. However, resolving protein subpopulations at the molecular level allows determination of the correct binding model that describes the nature of the protein system. For proteins with several binding sites, revealing discrete bound complexes would permit determination of the molecular stoichiometry and affinity within a single measurement. It would also identify inactive species and therefore have direct practical impact in supporting protein engineering

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Supporting Information Available. The supporting information contains the definition of the local reduced χ^2 used for model selection. For one antibody titration point, fits to the second cumulant are shown as an example of the effect of the model on the local reduced χ^2 . The supporting information also includes fits to cumulants from two data points from the Fab titration. This information is available free of charge via the internet at <http://pubs.acs.org>.

and purification efforts. In order to examine molecular heterogeneity, sensitive methods that can resolve multiple species must be developed and characterized.

Fluorescence fluctuation spectroscopy (FFS) probes a small volume in a solution (~ 1 fL) to measure properties from fluctuations due to individual molecules diffusing in and out of the sample volume. A number of analysis methods have been introduced to extract data from FFS experiments. The first and most widely used of these methods has been fluorescence correlation spectroscopy (FCS), which measures the temporal decay of fluctuations⁵. Provided the size of the molecular species differ significantly, FCS can be used to resolve free and bound populations⁶. When size cannot be utilized, more advanced approaches such as dual-color cross-correlation spectroscopy may be used to measure binding properties⁷. Using two or three different fluorescent probes, measurement of higher order complexes has been examined by employing the cross-correlation amplitude⁸⁻¹⁰. In practice, it is desirable to avoid the complexity of multiple fluorophores and detection channels by measuring concentration-dependent changes in the fluctuation amplitude of a single labeled ligand¹¹. However, the fluctuation amplitude alone is insufficient to directly resolve species, identify subpopulations and assign binding models.

We overcome this challenge by using an approach based on the distribution function of the photon counts.¹²⁻¹⁵ Distribution analysis utilizes statistically significant higher-order moments¹⁶ to identify species and their molecular brightness. It has been extended to include different sampling times, which improves signal-to-noise and provides sensitivity to diffusion rates¹⁷⁻¹⁸. Likewise, moment analysis calculates higher-order moments from the photon counts providing another method for resolving species^{16,19-21}. Closely related to the moments are the cumulants. Cumulants possess properties that are particularly well suited for the identification of multiple fluorescent species¹⁶. Cumulant analysis has also been extended to include measurement at different sampling times and called time-integrated fluorescence cumulant analysis (TIFCA)²². Using TIFCA allows resolution of multiple species by incorporating both the diffusion time and molecular brightness.

TIFCA was introduced to improve signal statistics and measure protein interactions in living cells²³. In vitro studies utilizing the sensitivity of TIFCA to resolve multiple bound species have yet to be completed. Here, we apply TIFCA to measure the stoichiometry of an immunoglobulin G (IgG) antibody by utilizing cumulants up to the fourth order. The IgG molecule contains two equivalent binding fragments or arms connected by a hinge region which allows flexible and independent movement²⁴⁻²⁶. Each antibody arm, called a Fab fragment, has one antigen binding site. The IgG molecule, therefore, provides a relatively simple system with higher order stoichiometry and practical application. We show that TIFCA allows resolution of two different bound species as well as the free ligand in solution using only a single color and establishes the binding model without a priori information.

We study the interaction between the monoclonal antibody (mAb) 106.3 and its ligand, a synthesized Alexa488 labeled peptide: Alexa488-BNP(5-13, C10A), which is the nine amino acid long epitope region of brain natriuretic peptide (BNP) ²⁷. mAb 106.3 is an antibody which may be used for detection of BNP in diagnostic assays for heart failure. The structure of Fab 106.3 in complex with BNP(5-13, C10A) was determined at 2.1 Å resolution and the system has been well characterized using fluorescence spectroscopy²⁸. FCS studies have been performed using BNP to establish the epitope and determine the affinity²⁷. This work showed that when bound to mAb 106.3, the Alexa488-BNP(5-13) fluorescence is partially quenched resulting in a change in molecular brightness. We first use TIFCA to confirm a single binding site in Fab 106.3 by resolving free ligand and single-bound Fab. We then apply TIFCA to a titration experiment performed with intact antibody and globally fit data to resolve concentrations of three species: free ligand, single-bound and

double-bound antibody. For both Fab and mAb, stoichiometry is confirmed by fitting concentration data to binding models. The fit also yields the affinity of the system.

2. Theoretical Background

TIFCA experimentally resolves subpopulations of the bound and free ligand present in titrations of ligand and antibody. The stoichiometry is determined by the number of species required to fit the TIFCA data. Each species is described by its own binding curve as a function of total antibody or ligand concentrations. Here we describe the expressions necessary to fit the concentrations of the subpopulations identified by TIFCA to two-species and three-species binding models.

2.1. Titration of Fab Fragments

In the first of three titration experiments presented below, fluorescently labeled ligand at a constant concentration $[L_t]$ is mixed with a successively increasing concentration of unlabelled Fab fragments. The subscript t denotes total concentrations of reagents in the reaction. Each titration point consists of different concentrations of free ligand $[L]$, free sites $[S]$ and bound ligand $[LS]$. The number of molecules in each state depends on the concentrations of Fab fragments and ligand as well as the dissociation constant. In a typical experiment of this nature, one usually determines the fraction of bound sites F_b which depends upon the dissociation constant K_D 29

$$F_b = P_b = \frac{K_D + [S_t] + [L_t] - \left((K_D + [L_t] + [S_t])^2 - 4[L_t] \times [S_t] \right)^{\frac{1}{2}}}{2[S_t]} \quad (1)$$

In Eq. (1), $[S_t]$ represents the total concentration of sites. The fraction of bound sites F_b is equal to the probability P_b that the binding site is occupied. The ligand exists in one of two states: the unbound state L and the bound state LS . The total concentration of the ligand in each state is described using the following relations

$$\begin{aligned} [L_t] &= [L] + [LS] \\ [S_t] &= [S] + [LS]. \end{aligned} \quad (2)$$

These equations can be combined with Eq. (1) to determine the concentrations of free and bound ligand:

$$[LS] = [S_t] \times P_b \quad (3)$$

and

$$[L] = [L_t] - [S_t] \times P_b. \quad (4)$$

Eqs. (3) and (4) predict the amount of ligand in each state as a function of the dissociation constant, total ligand and total sites.

2.2. Titration of antibody

For the case of two equivalent and independent binding sites in IgG26, the fraction of bound sites is given by Eq. (1), but with $[S_t] = 2[A_t]$, where $[A_t]$ is the total antibody concentration. We define three observable states for the ligand: the free ligand L and two bound forms (LA

and L_2A). The species LA specifies antibody with only a single binding site occupied, while L_2A characterizes antibody with both sites occupied by ligand. The total concentrations of the ligand and antibody are

$$\begin{aligned}[L_t] &= [L] + [LA] + 2[L_2A] \\ [A_t] &= [A] + [LA] + [L_2A].\end{aligned}\tag{5}$$

The right hand side of the first equality in Eq. (5) indicates the three ligand concentrations we wish to resolve experimentally. The probability $P_b = ([LA] + 2[L_2A]) / [S_t]$ that a given site of the antibody is occupied is equal to the fraction of bound sites, $P_b = F_b$. Thus, the concentration of free ligand $[L]$ is

$$[L] = [L_t] - [S_t] \times P_b.\tag{6}$$

Next, we need to distinguish between antibody with only a single site occupied and antibody with both sites occupied. Because the sites are independent, we use equal probability for binding to each site. The probability P_{one} , that only a single antibody site is occupied, is given by the product of the probability of one site being occupied and the probability of the other site being unoccupied:

$$P_{one} = 2(1 - P_b)P_b.\tag{7}$$

The factor of two in Eq. (7) accounts for the possibility of having either site occupied. Similarly, the probability P_{two} that both sites of the antibody are bound is,

$$P_{two} = P_b^2.\tag{8}$$

The concentration of antibody with a single occupied site is determined from the probability given by Eq. (7) multiplied by the total amount of antibody

$$[LA] = [A_t] \times P_{one}.\tag{9}$$

The concentration of antibody with both sites occupied is given by multiplying Eq. (8) by the total concentration of antibody

$$[L_2A] = [A_t] \times P_{two}.\tag{10}$$

Eqs. (6), (9) and (10) determine the concentration of the three fluorescent species ($[L]$, $[LA]$, $[L_2A]$) as a function of total antibody concentration.

2.3 Titration of ligand

Another method of measuring antibody stoichiometry is to maintain a constant concentration of sites and vary the concentration of the ligand. As before, the probability for an antibody to have one or two sites occupied is given by Eqs. (7) and (8). Therefore, the concentration of $[L]$, $[LA]$ and $[L_2A]$ follows Eqs. (6), (9) and (10).

2.4 Measurement of Free and Bound Ligand

In order to resolve species and determine the concentration of ligand in each of the states described above, physical properties that differentiate the species must be measured. Here we will focus on two properties that are measurable using fluorescence fluctuation spectroscopy (FFS). The first is the molecular brightness of the fluorescently labeled ligand. This is the average photon yield per molecule per unit time. For free ligand L , we define the molecular brightness as λ_L . For the antibody with a single ligand (LA), the brightness is λ_{LA} . The brightness of LA may or may not be equal to λ_L due to a possible quenching of the fluorophore upon binding. Therefore, $\lambda_{LA} = q\lambda_L$, where q is the quenching factor. The double-bound antibody L_2A contains two bound ligands and hence has the brightness $2\lambda_{LA}$.

The second parameter that can be measured with FFS is the diffusion time τ_D of the molecules as they pass through the excitation volume. If the size of the ligand is significantly smaller than antibody, the diffusion time changes substantially when bound. Therefore, in addition to brightness, the diffusion time of the molecules is also incorporated into the analysis. However, for binding of proteins of similar size, the diffusion time will not aid in resolving species. In such cases, the single and double bound states must be resolved using the change in molecular brightness alone.

The amount of each of the three species described above is measured using TIFCA, which determines the average number of molecules in the excitation volume with a given brightness and diffusion time. An overview of the method is provided in the appendix below. Please see the reference for a more detailed description²².

2.5 Fitting Binding Data

Fab titration data is described with a two-species model representing free ligand (L) and bound ligand (LS). TIFCA measures the number of free ligand molecules N_L and the number of ligand molecules bound to Fab fragments N_{LS} . A conversion factor ϕ is required to express concentrations as number of molecules in the observation volume. This conversion factor is calibrated independently before performing the titrations. Multiplication of the concentrations $[L]$ and $[LS]$ (Eqs. (3) and (4)) by ϕ yields the measured number of molecules

$$N_{LS} = \phi [S_t] \times P_b \quad (11)$$

and

$$N_L = \phi ([L_t] - [LS]) + N_L' \quad (12)$$

In Eq. (12), a parameter N_L' is added to account for any inactive ligand (or free dye) that may be present in the sample. This will be discussed in more detail below, however to be consistent with its physical meaning such an offset must not be included in the bound state described by Eq. (11). These two expressions will be simultaneously fit to the number of molecules determined from TIFCA.

For antibody titration experiments, a three-species model is used in the global fitting routine to represent the three species L , LA , and L_2A . The first two species are the same as for Fab fragments: the number of free ligand molecules, N_L , and the amount of single-bound antibody, $N_{Lb} = N_{LA}$. The third species is the amount of antibody with both sites bound, N_{L2A} . This value is multiplied by a factor of two in order to yield the number of ligand molecules associated with double-bound antibody, $N_{2b} = 2N_{L2A}$. Similar to the expressions shown for fitting titrations with Fab fragments, the conversion factor ϕ is again used with

Eq. (6), (9) and (10) to express the number of free ligand N_L , single-bound ligand N_{1b} and the number of ligand in the double-bound state N_{2b} , which yields the following expressions

$$N_L = \phi ([L_t] - [S_t] \times P_b) + N_L', \quad (13)$$

$$N_{1b} = \phi [A_t] \times P_{one} \quad (14)$$

and

$$N_{2b} = \phi [S_t] \times P_{two}. \quad (15)$$

3. Results

3.1 Instrument calibration

It is known that the theoretical point spread function (PSF) used for modeling FFS data only approximately describes the experimental PSF³⁰⁻³². Deviations become more pronounced as higher-order moments are calculated. For this reason, it is necessary to first calibrate the instrument using a sample which is known to contain a single species. This calibration step provides experimentally determined values of the PSF-dependent shape factors γ_3 and γ_4 , while γ_1 and γ_2 are based on a three-dimensional Gaussian PSF (See Eq. (A1)). We used a solution of 16 nM Alexa488 for calibration. Data was taken with ~ 3.5 mW on the sample at a frequency of 50 kHz and rebinned eight times by consecutive factors of two, which corresponds to sampling times ranging from 20 μ s to 2.6 ms. The first four cumulants for each bin time were calculated and fit as shown in Fig. 1. Each panel displays a single normalized cumulant κ_r/T^r plotted as a function of bin time. The fitted brightness is 44 kHz, the diffusion time is 0.047 ms and $N = 2.8$ molecules. The calibration parameters returned by the fit are $\gamma_3 = 0.25$ and $\gamma_4 = 0.22$. The calibration parameter ϕ which relates concentration and number of molecules in the observation volume is given by $\phi = 2.8 / 16$ nM = 0.18 molecules / nM.

3.2 Fab Titration

Fab 106.3 was added to solutions of 20 nM A-BNP(5-13) at concentrations ranging from micromolar to subnanomolar. Data from each of the samples was acquired at 50 kHz for 100 seconds. The data was then successively rebinned by factors of two from 50 kHz to 390 Hz and the first four factorial cumulants at each bin time were calculated. For each titration point, the total intensity of the sample decreases as Fab fragments are added to the solution, indicating binding induced quenching. If A-BNP(5-13) is completely quenched upon binding, a single species representing free ligand would provide a sufficient description. To explore this, we first globally fit cumulants from the titration data using a single-species model. In the fit, the brightness and diffusion time remain constant for each titration point while the number of molecules varies. The single species model does not fit this data (reduced $\chi^2 = 420$). Therefore, the cumulants were globally fit (reduced $\chi^2 = 2.4$) using a two species model by linking the brightness and the diffusion time of each species. Only the number of molecules of each of the two species was allowed to vary. One of the two species identified by TIFCA corresponds to free A-BNP(5-13) with a brightness of 47.8 kHz and a diffusion time of 0.07 ms. The other species corresponds to bound A-BNP(5-13) with a brightness of 8.1 kHz and a diffusion time of 0.19 ms. Two sets of cumulants and corresponding fits for two points from this titration data are described in the supplementary materials and shown in Fig S2.

The global TIFCA fit identifies the concentration dependent population of the free and bound ligand (Fig. 2). The number of molecules of the bound ligand increases with the Fab 106.3 concentration, while the amount of free A-BNP(5-13) decreases, as expected for a binding titration experiment. However, the number of free ligand molecules becomes constant at high Fab concentrations. This result instantly tells us that the experiment contains a fluorescent species that is unable to bind to the antibody. This additional species has the same brightness and diffusion time as the free ligand. Further analysis regarding the nature of this species will be presented later. We now fit the binding curves of Fig. 2 globally to a single site binding model which includes the presence of the inactive species (Eqs. (11) and (12)). The fit (reduced $\chi^2 = 1.4$) yielded a total active ligand concentration of 15.6 ± 0.1 nM, a dissociation constant K_D of 0.10 ± 0.06 nM, and an unbound ligand concentration of 2.5 ± 0.1 nM.

3.3 mAb Titration

A titration using mAb 106.3 was performed in the same manner as described above using 30 nM of A-BNP(5-13), the concentration of mAb 106.3 was varied from 500 nM to subnanomolar. The first four cumulants of each measurement were calculated for global fitting with TIFCA. Although we know that an IgG antibody has two sites and requires a three-species model, we examined the ability of TIFCA to establish the correct binding model by comparing analysis using both a two-species and three-species model. Globally fitting TIFCA data to the two-species model (only one bound species) yielded a global reduced $\chi^2 = 4.0$, while the three-species model returned $\chi^2 = 1.9$. Although the three-species model leads to a better description than the two-species model, the difference in the global reduced χ^2 values alone is not sufficient to select one model over the other. Thus, we examined the local reduced χ^2 values (see supplementary materials for a definition of the local reduced χ^2) of the individual cumulants within the global fit and found that the second cumulant is most sensitive to the model. This is most evident in samples from the titration in which all three species are present. Specifically, at titration points containing 4nM to 60nM of antibody, reduced χ^2 values range from 2 to 78 using the two-species model while ranging from 0.4 to 3 using the three-species model. The average local reduced χ^2 for the second cumulant of all titration points is 9.1 for the two-species and 1.5 for the three-species model. Hence, the two-species model is ruled out by the poor fit of the second cumulant. Figure S1 in the supplementary material shows the effect on one titration point in this experiment. Due to their larger uncertainty, the higher order cumulants are less sensitive to the model than the second cumulant. To further test the model, an attempt to fit the number of molecules in each state with binding curves described by a single site was made (Eqs. (11) and (12)), but resulted in a poor fit with a reduced $\chi^2 = 20$. Therefore, the single site binding model is ruled out by both fitting procedures and the two-site model is selected.

The global TIFCA fit to the three-species model determined $\lambda_L = 45$ kHz and $\lambda_{LA} = 6.2$ kHz ($\lambda_{2LA} = 12.4$ kHz). The diffusion times are 0.07 ms for free A-BNP(5-13) and 0.69 ms for either of the two bound states. Table 1 displays the global fit values for the number of molecules from three concentrations of mAb. When only a very small amount of antibody is present (mAb = 0.24nM), ligand molecules are in large excess, which results in the absence of the single-bound species. At a higher mAb concentration (mAb = 31.5nM), the amount of sites is on the order of the amount of ligand and all three states are populated. For reference, the fits to the four cumulants from the titration point with 31.5 nM mAb is shown in Fig. 3. Finally, at the last titration point in Table 1 (mAb = 500 nM) there are no antibody molecules with both sites occupied ($N_{2b} = 0$), because binding sites are in a large excess. The uncertainty listed in Table 1 is calculated by fitting segments of data as separate experiments as described in the experimental methods section.

Fig. 4 shows the number of ligand molecules in each of the three states as a function of antibody concentration. The curves follow the behavior expected for a two-site system with equal binding probability. Initially, both sites are occupied until there are more sites than ligand present. Therefore, the amount of ligand associated with double-bound antibody (L_2A , circles) increases before decreasing as antibody is added to the solution. The lines in Fig. 4 represent a global fit (reduced $\chi^2 = 2.3$) of the data to Eqs. (13), (14) and (15). The fitted dissociation constant $K_d = 0.7 \pm 0.2$ nM. The amount of active ligand available for binding is 24.2 ± 0.7 nM. As in the case of Fab, a nonreactive fluorescent species with a concentration of 3.9 ± 0.2 nM was identified, which is $\sim 14\%$ of the total ligand concentration (28 ± 1 nM).

3.4 Ligand Titration

As a final experiment we performed a titration of A-BNP(5-13) using a constant antibody concentration of 5 nM. Nine measurements were taken as the concentration of A-BNP(5-13) varied from 1 to 25 nM. Following the same procedures described above, cumulants were globally fit to both a two-species and three-species model yielding a global reduced χ^2 of 2.0 and 1.2, respectively. As before, the global reduced χ^2 are too similar to unequivocally determine the model. Therefore, we again examined the local reduced χ^2 of the second cumulant to establish the better fit. Using a two-species model, the local reduced χ^2 of the second cumulant has an average of 5.0 with a maximum of 9.0. The three-species model yields an average reduced $\chi^2 = 1.1$ with a maximum local value of 2.7. As before, the local values for the second cumulant indicate a two-site system. Furthermore, an attempt to fit the number of molecules to a two-species model (Eqs (11) and (12)) resulted in reduced $\chi^2 = 10.4$.

The fit using the three-species model determined a brightness of 48 kHz for the free ligand L and a brightness of 6.2 kHz for the single-bound state LA . The diffusion time for the free ligand L is 0.06 ms and 0.72 ms for the antibody in either state (LA or L_2A). In this case, the brightness and diffusion time are consistent with the values determined in the abovementioned antibody titration experiment. However, fitting ligand titration data with the two-species model yielded a brightness of 5.6 kHz and a diffusion time of 1.5 ms for the bound state, both of which are not consistent with the values measured in the antibody titration. This result is further confirmation of the selected binding model.

The number of molecules of A-BNP(5-13) in each of the three states from a three-species fit are plotted in Fig. 5. The amount of single-bound ligand (triangles) increases initially, and begins to decrease as ligand is added and sites begin to fill. Therefore, a corresponding increase in double-bound antibody is observed (circles). Also, the amount of free ligand continually increases as the number of total ligand surpasses the total sites (diamonds). The lines shown in the plot are a global fit to Eqs. (13), (14) and (15). The fit, with reduced $\chi^2 = 2.0$, arrived at a total concentration of 6.4 ± 0.1 nM antibody with a dissociation constant $K_d = 0.6 \pm 0.1$ nM for the system. As with the earlier titrations, the presence of $\sim 11\%$ inactive fluorescent species was identified.

3.5 Serial Dilution

Another method for performing binding titrations involves serial dilution of a mixture containing equal concentrations of antibody sites and ligand. As in the previous experiments, the number of species required to fit the data indicates stoichiometry. Calculation of the fraction of free ligand and antibody with either one or two sites occupied as a function of concentration can be used to determine affinity. Model curves based on this principle are shown in Fig. 6. A dissociation constant of $K_D = 0.5$ nM is assumed, and the fraction of each species is plotted as a function of total antibody concentration. The

dissociation constant corresponds to the intersection of the fractions of single-bound (dashed line) and double-bound (dotted line) antibody. To generate the presence of all three species, this method requires concentrations of both ligand and antibody on the order of K_D . For A-BNP(5-13) and mAb 106.3 background fluorescence sets a lower concentration limit of ~ 1 nM. Therefore, this approach was not pursued in this study. However, for systems with lower affinity ($K_D \sim 1 \mu\text{M}$), the technique may prove useful for characterizing both stoichiometry and affinity because experiments with higher concentrations can be performed.

4. Discussion

We have applied TIFCA to resolve three sub-populations within antibody binding titrations using only a single color. Furthermore we found that TIFCA allows selection of the proper binding model as well as measurement of the stoichiometry in solution. For titration data with isolated Fab fragments, TIFCA alone was sufficient to validate the introduction of a bound species. For whole antibody, we first fit titrations to a two-species model, which does not include the presence of double-bound molecules. Introduction of a third species resulted in a decrease of the local reduced χ^2 of the second cumulant, by a factor of five or more, but had a lesser effect on the global reduced χ^2 due to the higher uncertainties in the third and fourth cumulants. Therefore, examining the local reduced χ^2 of the second cumulant provides a sensitive means to establish the correct number of species in the binding model. Confirmation of the selected model is accomplished in the next step by fitting the number of molecules of each species as a function of concentration. We showed that this procedure identifies the proper model without prior knowledge of the molecular brightness and diffusion time.

It was earlier mentioned that the IgG molecule contains two equivalent Fab fragments connected by a hinge region which allows flexible and independent movements. Therefore, binding of relatively small soluble ligands to bivalent antibody molecules follows a simple binding model which treats each site as fully independent, as was observed in the current system. In a hypothetical case with larger antigens that cause partial hindrance of the second site upon binding to the first, the simple model applied here will fail. Consequently, fitting binding curves will require a different model that accounts for such effect.

The concentrations of the reagents in the experiments presented in Figs. 2, 4 and 5 were optimized for determination of stoichiometry of the antibody complexes. Determining affinity at stoichiometric conditions is typically avoided because the resulting binding plot predominantly depends on the concentration of ligand and sites, not on the affinity of the system 29. However, to confirm the model selection, we globally fit the number of molecules of each species, which yields an estimate of the dissociation constant. The values returned from the three experiments are 0.10 ± 0.06 , 0.7 ± 0.2 and 0.6 ± 0.1 nM indicating the K_D range of 0.04 - 0.9 nM. The dissociation constant $K_D = 0.4 \pm 0.1$ nM was previously obtained for mAb 106.3 and A-BNP(5-13) at optimal equilibrium conditions²⁷.

In this study, we also found that TIFCA enables identification of inactive components in reaction mixtures. Although the peptide was initially purified using reverse-phase HPLC, all binding curves generated using TIFCA revealed the presence of unbound A-BNP(5-13) under conditions in which all antigen molecules must be bound. In a separate measurement of A-BNP(5-13) saturated with mAb we observed $\sim 12\%$ of unbound ligand. This sample was then measured after passing through a size exclusion column (NAP-5, GE Healthcare), which reduced the amount of unbound A-BNP(5-13) to $\sim 1\%$. This result confirms that even after purification some free ligand species remains in the sample. The amount of inactive labeled peptide partially contributes to the difference in the binding induced quenching of A-

BNP(5-13) when compared to previous labeled peptide preparations²⁸, while other source of variations in quenching are yet to be determined. However, the presence of this inactive fraction does not restrict the ability of TIFCA to resolve the free, single and double-bound species because such inclusion does not require additional fitting parameters beyond the three-species model. It is important to emphasize that only measurements in which absolute concentrations of each species are determined are able to resolve inactive antigen. They can be performed independent of or in addition to advanced separation techniques. Thus, TIFCA can aid in assessment of antigen activity in a given antibody-antigen system. Other techniques, such as fluorescence quenching or anisotropy, would not be able to reveal this inactive fraction because they require complete saturation to accurately define the bound state.

Characterizing antibody-antigen interactions in the detail described here has important practical implications. Antibodies are widely utilized in medical diagnostics to detect the concentration of a particular antigen in a patient sample. The antibody 106.3 found application in a diagnostic BNP test²⁷. Also, progress in antibody engineering has led to the development of numerous antibody constructs for therapeutic applications including treatment of autoimmune diseases and cancer³³⁻³⁴. More recently, bi-specific dual-variable-domain antibodies with four binding sites have been produced³⁵. These constructs have two sites for each target antigen giving a total stoichiometry of four. Although the binding models presented in this work are not applicable for such systems, TIFCA may prove useful in resolving bound states and measuring the more complicated stoichiometries.

In conclusion, TIFCA provides a powerful biophysical tool for probing the properties of protein-ligand interactions directly without the need for separation steps or other procedures which may disrupt binding equilibrium. By exploiting the improved signal statistics, TIFCA enables separation of subpopulations within a mixture using only a single fluorescent species, which has not been achieved with other methods. By measuring the concentrations of molecular subpopulations, TIFCA offers determination of the stoichiometry, model selection and estimation of affinity in a single titration experiment. We believe that this method will find applications in studying protein systems with even more complex binding properties.

5. Experimental Methods

5.1 Antibody and Peptide Samples

The monoclonal antibody 106.3 (IgG1, κ) and its Fab fragments were obtained as described previously^{27,28}. Concentrations of antibody and Fab fragments were measured on a Cary 4 spectrophotometer (Varian, Sugarland, TX) using an extinction coefficient of $\epsilon_{279} = 217,000$ (M cm)⁻¹ and $\epsilon_{279} = 72,500$ (M cm)⁻¹, respectively. Better than 95% purity of the proteins was confirmed by SDS-PAGE and HPLC using a TSK G3000SW_x column (Tosoh, Japan).

The fluorescently labeled short peptide, the ligand for mAb 106.3, 6-Alexa488-BNP(5-13, C10A), was prepared as described²⁷. The concentration of the peptide was determined using $\epsilon_{515} = 71,000$ (M cm)⁻¹ as quoted by the Alexa488 manufacturer (Invitrogen, Carlsbad, CA). This peptide will be referenced as A-BNP(5-13) throughout this manuscript.

5.2 Fluorescence measurements

Experiments were performed on a two-photon FFS microscope coupled with an ALBA correlation spectrometer (ISS, Champaign, IL) for data acquisition. A mode-locked titanium-sapphire laser (Tsunami, Spectra Physics, Mountain View, CA) pumped with an intracavity doubled Nd:YVO₄ (Millenia VIs, Spectra Physics) serves as the infrared excitation source. The beam is expanded 4X with a beam expander (Newport Corp., Irvine,

CA) before passing through the back aperture of a Nikon Plan Apo 60X/1.2 NA objective on a Nikon TE300 fluorescence microscope (Nikon, Kanagawa, Japan). A dichroic mirror (700DCSPXR, Chroma, Rockingham, VT) directs excitation light into the objective for focusing on the sample. Emitted fluorescence is collected by the same objective and is further filtered by a two-photon band pass filter (E700sp-2p, Chroma) before passing into the ALBA correlation spectrometer. Inside the ALBA correlation spectrometer the emitted fluorescence is focused onto a single-photon counting avalanche photodiode (SPCM-AQR-15, Perkin-Elmer, Vaudereuil, Canada).

5.3 Data analysis

Raw photon count data was collected using the acquisition software provided by the ALBA manufacturer (ISS Vista, version 3.12). The data was exported for analysis using programs written for IDL version 6.4 (ITT Corp., Boulder CO). First, rebinned fluorescence cumulants along with their variances were calculated from the raw data up to fourth order for a range of bin times T and analyzed as described²². For a single measurement, the four cumulants were fit simultaneously, following Eq. (S1) (Supplementary Information), to obtain the value for the brightness, number of molecules and diffusion time of each species. To fit data acquired for each sample in a titration experiment, a global fitting routine, using a multi-species model was employed in which the diffusion time and brightness of each species were linked. Only the number of molecules of each species was allowed to vary across the titration points. In order to estimate the uncertainty in the number of molecules returned from titration experiments, the raw photon count data from a single experiment was divided into shorter data segments. Each segment was treated as a separate experiment and global TIFCA fitting was performed using the cumulants calculated for each segment. The results were used to estimate the standard deviation of the TIFCA fit parameters. The standard deviation of the fitted number of molecules for each species was then used to perform weighted fits to the binding curves and determine the dissociation constant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

This section provides a general overview of TIFCA. A more detailed description can be found in the literature²².

Fluorescence cumulant analysis was initially developed to determine photon count moments together with their uncertainty in order to provide quantitative modeling with a rigorous quality of fit criterion¹⁶. The r^{th} -order factorial cumulant $\kappa_{[r]}$ of the photon counts in the limit of short sampling times ($T \ll \tau_D$) is given by

$$\kappa_{[r]} = \gamma_r (\lambda T)^r \langle N \rangle \quad (\text{A1})$$

where λ is the molecular brightness and $\langle N \rangle$ is the average number of molecules of the fluorescent species. The quantity γ_r is a point spread function (PSF) dependent shape factor

and has been described previously^{16,36}. The factorial cumulants of a mixture of M species is given by the sum over the cumulants of each species:

$$K_{[r]}^M = \sum_{j=1}^M K_{[r]j}. \quad (\text{A2})$$

The simple additive nature is very useful for separating molecular species. We will frequently refer to the factorial cumulant $K_{[r]}$ simply as cumulant.

The determination of higher-order cumulants requires a sufficiently high signal-to-noise ratio (SNR), which is often not achieved in the short sampling time limit. To address this challenge, cumulant theory was extended to cover all sampling times²². While Eq. (A1) is restricted to short sampling times, the general equation describing cumulants for arbitrary sampling times is given by

$$\tilde{K}_{[r]} = \gamma_r \lambda^r \langle N \rangle B_r(T, \tau_D), \quad (\text{A3})$$

where the factor T^r in Eq. (A3) has been replaced by the r -th binning function $B_r(T, \tau_D)$, which depends on the diffusion time τ_D as described in detail elsewhere²². Eq. (A3) impacts data analysis in two specific ways. First, longer sampling times significantly improve the SNR of higher-order cumulants. Second, the cumulant depends on the diffusion time, which allows the simultaneous determination of brightness and diffusion information from the sample. In order to take full advantage of Eq. (A3), experimental cumulants $K_{[r]}(T)$ are calculated for a range of sampling times and fit to the time-dependent cumulant functions of Eq. (A3). Cumulants for different values of T are conveniently determined by rebinning of the original photon count sequence to longer sampling times as described previously²².

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Figure 1.

Instrument calibration. The first four cumulants and the resulting fit from a measured solution of 16 nM Alexa488. The brightness determined by the fit is 44 kHz, the number of molecules is 2.8 and the diffusion time is 0.047 ms (reduced $\chi^2 = 1.0$). The calibrated beam shape parameters are $\gamma_3 = 0.25$ and $\gamma_4 = 0.22$ with $\gamma_2 = 0.3535$ fixed during the fit.

Figure 2.

Number of molecules from a titration of 20 nM A-BNP(5-13) with Fab. A global fit (dashed and solid line) of the number of molecules to Eqs. (11) and (12) determined a dissociation constant of 0.10 ± 0.06 nM (reduced $\chi^2 = 1.4$). The inset shows the same data plotted on a log-linear scale. The last three titration points are only shown in the inset in order to best present the data in the main panel.

Figure 3. TIFCA results from globally fitting titration data with 30 nM A-BNP(5-13) and mAb 106.3. The figure shows the fitted cumulants for 31.5 nM mAb. The number of molecules determined from the fit are listed in Table 1.

Figure 4.

Titration of 30 nM A-BNP(5-13) with mAb 106.3. The number of molecules of free ligand (diamond), ligand associated with single-bound antibody (triangle) and ligand associated with double-bound antibody (circles) versus antibody concentration is shown. The inset shows the data on a log-linear scale. The lines represent the result of a global fit (reduced $\chi^2 = 2.3$) of the titration, which recovered a dissociation constant of 0.7 ± 0.2 nM.

Figure 5.

Titration of ligand against a constant concentration of antibody. The number of molecules of free ligand (diamond), ligand associated with single-bound antibody (triangle) and ligand associated with double-bound antibody (circles) versus ligand concentration is shown. The lines represent the result of a global fit to the data yielding a dissociation constant of $K_D = 0.6 \pm 0.1$ nM (reduced $\chi^2 = 2.0$).

Figure 6.

Model of expected values for serial dilution. The plot indicates the expected fraction of ligand in each of the three possible states for the case when equimolar concentrations of antibody sites and ligand are serially diluted. The dissociation constant is 0.5 nM. The location of the intersection between the single-bound (dashed line) and double-bound ligand (dotted line) at 0.5 nM is an indicator of the affinity.

Table 1

Number of molecules of each ligand species determined by TIFCA analysis at three antibody concentrations.

mAb (nM)	N_L	N_{Ib}	N_{2b}
0.24	3.6 ± 0.1	0 ± 0.2	1.2 ± 0.7
31.5	0.66 ± 0.02	2.3 ± 0.2	0.9 ± 0.2
500	0.50 ± 0.02	3.6 ± 0.2	0 ± 0.02