Model-independent counting of molecules in single-molecule localization microscopy

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ABSTRACT Most biomolecular processes rely on tightly controlled stoichiometries, from the formation of molecular assemblies to cellular signaling. Single-molecule localization microscopy studies of fluorophore blinking offer a promising route to probe oligomeric states. Here we show that the distribution of the number of blinking events assumes a universal functional form, independent of photophysics, under relatively mild assumptions. The number of photophysical states, the kinetics of interconversion, and the fraction of active fluorophores enter as two or three constants. This essentially model-independent formulation allows us to determine molecule counts from fluorophore blinking statistics. The formulas hold even if the fluorophores have many different yet unresolved dark states, as long as there is only a single fluorescent state, or if there are different yet resolvable fluorescent states, as long as there is only a single dark state. We demonstrate the practical applicability of this approach by quantifying the oligomerization states of membrane proteins tagged with the mEos2 fluorescent protein. We find that the model parameters, obtained by likelihood maximization, are transferable. With the counting statistics being independent of the detailed photophysics and its parameters being transferable, the method should be robust and broadly applicable to counting colocalized molecules in vivo and in vitro.

INTRODUCTION

Knowledge of assembly and subunit architecture of macromolecular complexes in a cellular context is essential to inferring their biological function. Fluorescence microscopy has become increasingly popular for quantifying molecular numbers in the near-native cell environment, as it forgoes invasive protein preparation or isolation procedures (Coffman and Wu, 2014; Fricke et al., 2015b). However, at high protein densities, the spatial resolution limit of ~200 nm in conventional microscopy hampers direct observation of single-protein complexes. Superresolution fluorescence techniques present a powerful solution to bypass this limit. Among these, single-molecule localization microscopy (SMLM) is particularly well suited, as it is based on the detection of single emitters. By separating fluorescence emissions in time, individual fluorophores can be localized with high precision and ultimately provide a fluorescence image with spatial resolution improved by roughly an order of magnitude (Sahl and Moerner, 2013). The single-molecule method is commonly realized with photoswitchable or photoactivatable fluorescent probes and particularly useful for molecular counting applications, since fluorescence emission events relate to the number of underlying molecules (Fürstenberg and Heilemann, 2013; Figure 1). A straightforward analysis is challenged, however, by the photophysical properties of the fluorophores. Reversible transitions into nonfluorescent dark states lead to multiple fluorescence bursts of the same fluorescent probe, such that a simple counting of bursts would overestimate molecular numbers (Heilemann et al., 2005; Greenfield et al., 2009; Annibale et al., 2011a; Sengupta et al., 2011; van de Linde and Sauer, 2014).

Various approaches have been developed to correct for overcounting caused by fluorescence intermittency (“blinking”). A straightforward estimate of molecular numbers is obtained by taking into account the average number of blinking events of the fluorescent probe (Lando et al., 2012; Endesfelder et al., 2013; Ehmann et al., 2014; Letschert et al., 2014; Löschberger et al., 2014;
Ricci et al., 2015). Particularly promising counting strategies involve photoactivatable fluorescent proteins (PAFPs) for direct and stoichiometric protein labeling. Their photokinetics, after activation, is generally assumed to obey a simple three-state model in which fluorophores can cycle between a dark and a fluorescent state before irreversible photobleaching (Annibale et al., 2010; Coltharp et al., 2012). A widely used counting method considers spatially clustered fluorescence bursts that occur within a characteristic temporal threshold as one molecular count (Greenfield et al., 2009; Annibale et al., 2011b; Lehmann et al., 2011). Lee et al. (2012) pioneered a refined approach in which the kinetic equations are solved to obtain the photokinetic parameters of PAFPs and in turn a more robust estimate of molecular counts (Lee et al., 2012; Avilov et al., 2014). An elegant extension treats the molecular photokinetics directly with continuous-time aggregated Markov models (Rollins et al., 2015).

Most approaches assume a simple three-state kinetic model of fluorescence photophysics, in which, after activation the fluorescent (F), dark (D), and bleached (B) states interconvert according to F → B and F ⇄ D. However, the photokinetics of the fluorescent probes is often not known in sufficient detail and may turn out to be too complex for exact kinetic modeling (Huang et al., 2006; Yeow et al., 2006; Widengren et al., 2007; Vogelsang et al., 2008; Kottke et al., 2010). Furthermore, concepts based on dark-state thresholding are not applicable to photoswitchable fluorescent probes with prolonged dark-state dwell times, where photoswitching cycles may overlap (van de Linde and Sauer, 2014). Finally, it may not be necessary to model the photophysical kinetics in detail to extract molecule counts.

Here we introduce a simple approach to counting molecules by demonstrating that the functional form of the blinking statistics is indeed independent of photophysics under relatively mild assumptions. Therefore our approach does not require knowledge of photophysical states, their connectivity, their relative populations, the associated photokinetic rates, or temporal information on photoswitching events. All photophysical effects are condensed into at most three parameters in the distribution of the number of blinking events, given the number of fluorophores. The resulting analytical formulas for the blinking statistics provide the basis for a model-independent and robust estimate of the number of colocalized molecules from single-molecule microscopy data.

In Materials and Methods, we derive the general expression for the probability distribution of single-molecule transition counts. We then develop formulas for fluorophore blinking statistics. For simple kinetic models of the photophysics, we derive explicit expressions for the model parameters. In Results, we illustrate the practical application of the theory by determining molecule counts for fusion constructs of mEos2 with membrane proteins.

**RESULTS**

We apply the molecule-counting formalism derived in Materials and Methods to five SMLM experiments. These experiments used the mEos2 protein as fluorescent tag for different membrane proteins expressed in HeLa cells. The SMLM counting data have been previously published, and the experimental procedures, such as sample preparation, SMLM microscopy, and data analysis, are fully described elsewhere (Fricke et al., 2015a). Having used the same fluorophore in multiple experiments allows us to infer p and q from data for systems with m = 0 and 1 and then determine the oligomerization state of the other complexes. In the final example, we show that one can also estimate monomer/dimer ratios.

**Single mEos2**

Using blinking statistics data obtained for single-molecule surfaces of bacterially expressed and purified mEos2 (Fricke et al., 2015a), we first show that mEos2 exhibits simple blinking statistics (Eq. 12) for p = p₀. Figure 2A compares the blinking statistics of isolated mEos2 observed and calculated for the maximum-likelihood estimate of p = 0.289 ± 0.010. Errors are estimated by bootstrapping (i.e., from repeated maximum-likelihood optimizations for counts redrawn with replacement from the observed statistics, c(n)). For the two-parameter description (Eq. 9), the maximum-likelihood estimates

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**FIGURE 1:** Molecule counting in single-molecule localization microscopy. From the time-ordered series of total internal reflection images used to create the SMLM image (left; scale bar, 500 nm), one determines fluorescence intensity traces at particular spots (boxes) as a function of time. The number n of blinking events in these traces is counted (middle). The number of colocalized molecules is then determined from the frequency distribution c(n) of the number n of blinking events (right) by comparison to predictions for different oligomerization states (lines).

**FIGURE 2:** Extraction of photophysical parameters from blinking statistics of (A) mEos2 monomers and (B, C) CTLA-4 dimers. The measured counts (Fricke et al., 2015a) c(n) are shown as boxes. (A) For the mEos2 monomer, the maximum-likelihood fits for simple counting statistics (Eq. 11; thick green line; p = p₀ = 0.289) and for the more complex two-parameter statistics (Eq. 7; thin blue line; p = 0.286 and p₀ = 0.295) are nearly indistinguishable. (B) For the CTLA-4 dimer, we obtain a value of q = 0.295 with p = p₀ = 0.289 fixed. Insets, cumulative distribution functions (experiment: symbols; theory: lines). (C) Isocontour lines of the log-likelihood in simultaneous fits of p and q to CTLA-4 count statistics (solid/thin lines: contours −1/−2 relative to maximum). Δ indicates the difference of the respective maximum of the log-likelihood relative to the dimer model.
FIGURE 3: Blinking statistics of (A) CD86-mEos2, (B) VSVG-mEos2, and (C) CD80-mEos2. Boxes show experimental counts (Fricke et al., 2015a). Lines show the predicted statistics for monomers (green), dimers (blue), trimers (gold), and tetramers (dark blue; only VSVG), with ρ and q fixed at the values obtained for single mEos2 and CTLA-4 dimers. For CD80, a mixture of 45.5% monomer and 54.5% dimer provides the best fit (black line). Insets, cumulative distribution functions (experiment: symbols; theory: lines).

are \( p = 0.286 \pm 0.012 \) and \( p_0 = 0.296 \pm 0.01 \), that is, very close to each other and the estimate of \( p \) for the simpler form of \( p_0(m) \) (Eq. 12). Indeed, the gain in log-likelihood of 0.1 log units over the one-parameter description is far below the Bayes information criterion (BIC) of 3.2 to justify this more complex description.

CTLA-4 dimer

With \( p \) determined from the single-mEos2 data, we extracted \( q \) from data (Fricke et al., 2015a) for the covalent dimeric cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) with two mEos2 fluorophores attached per dimer and expressed and imaged in HeLa cells. The dimeric state of CTLA-4 is well established (Fricke et al., 2015a). Figure 2B compares the observed and calculated blinking statistics for CTLA-4, where the one free parameter, \( q = 0.295 \pm 0.040 \), was determined by maximizing the likelihood for fixed \( p = 0.289 \pm 0.010 \), using \( p_0(m) \) from Eq. 13 for the simple statistics. In other words, ~70% of mEos2 molecules are active and photodetected on average, which is in reasonable agreement with previously determined values (Puchner et al., 2013; Durisic et al., 2014).

Figure 2C shows isocontour lines of the log likelihood for simultaneous fits of \( p \) and \( q \). For the dimer, the isocontour lines form ellipses whose main axes are closely aligned with the \( p \) and \( q \) axes, indicating that the two parameters are nearly independent in the fit. Whereas the dimer model is clearly superior to the monomer, with a difference of >20 log units in the log-likelihood, the trimer and tetramer models are lower by only 2.0 and 2.6 log units, respectively. However, these fits required unrealistically high fractions \( q \) of damaged molecules, \( q > 0.6 \), consistent with an expected degeneracy between \( m \) and \( q \). Calibrating \( q \) properly is thus an important prerequisite for accurate molecule counts.

CD86 monomer

Figure 3A compares the observed blinking statistics for CD86 monomers fused to mEos2 (Fricke et al., 2015a) to the predictions for \( m = 0, 1, \) and 2 with fixed \( p = 0.289 \) and \( q = 0.295 \). Visually, only the monomer, \( m = 0 \), fits the data. Indeed, CD86 is expected to be monomeric (Fricke et al., 2015a). This expectation is supported by a more quantitative statistical analysis, with the log-likelihoods of the dimer and trimer being lower than the monomer value by >200 and almost 700 log units, respectively.

Vesicular stomatitis virus glycoprotein (VSVG)

Figure 3B compares the blinking statistics observed for trimers of vesicular stomatitis virus glycoprotein (VSVG) fused to mEos2 (Fricke et al., 2015a) to the predictions up to tetramers, with fixed \( p = 0.289 \) with a fraction of \( w = 0.455 \pm 0.05 \) monomer and the rest dimer explains the observed statistics well. We determined the relative weights by maximizing the likelihood with respect to \( w \) for \( p(n) = w p_0(n) + (1 - w) p_1(n) \). The log-likelihood of the monomer/dimer mixture exceeds those of the monomer and dimer alone by ~50–70 log units, compared with a BIC of 6.7. Using a mixture with one additional parameter, \( w \), is thus justifiable. This finding is consistent with an analysis of earlier studies (Fricke et al., 2015a) that suggested coexistence of monomers and dimers at the plasma membrane.

Statistical accuracy of molecule counts

In Figure 4, we assess the statistical accuracy of estimated molecule counts. We repeatedly picked \( N_{\text{spot}} = 10 \) and 100 spots for analysis at random from the 856 CD86 and 411 VSVG spots and estimated the count \( M = m + 1 \) from the resampled \( c(n) \) by determining the maximum of \( L \) in Eq. 17 over \( m \). For the monomeric CD86 and the trimeric VSVG, we find that 100 spots produce the correct estimate in >99.9 and 95% of the resampled cases, respectively (Figure 4A). To estimate the accuracy also for larger oligomers of size \( M_0 = m_0 + 1 \), we repeatedly sampled blinking counts \( c(n) \) according to \( p(n|m) \) for mEos2 parameters \( p = p_0 = 0.289 \) and \( q = 0.295 \). Under idealized assumptions of only counting noise in \( c(n) \), the maximum-likelihood estimates of \( M \) are in excellent agreement with \( M_0 \) for as few as 100 spots (Figure 4B).

FIGURE 4: Accuracy of estimated molecule counts \( M \) for different oligomer sizes \( M_0 \) and numbers of spots \( N_{\text{spot}} \) analyzed. \( p(M|M_0) \) is the distribution of \( M = m + 1 \) maximizing the likelihood (Eq. 17) in 105 repeated samplings of blinking statistics \( c(n) \). (A) Resampled experimental counts for CD86 and VSVG fused to mEos2. (B) Synthetic data for \( M_0 = 1, 5, 10, 15, \) and 20 mEos2 tags (left to right) in \( N_{\text{spot}} = 10, 100, 1000 \) spots (blue). Black vertical lines indicate exact values \( M_0 \). Dashed lines in B show the probability \( p(M_0 | M_0) \) of estimating \( M_0 \) exactly for different \( N_{\text{spot}} \).
**DISCUSSION**

We derived exact expressions for the distribution $p_m(n)$ of the number $n$ of blinking events in SMLM for different numbers $m$ of 1+1 localized fluorophores. By using a generating function approach, we showed that the functional form of $p_m(n)$ does not depend on the photophysics under relatively mild assumptions. For a wide class of photophysical models, the form of the count statistics is independent of the number of photophysical states, their connectivity network, and the exact time dependence. The main requirement is that transitions between microscopic states are Markovian, that is, memoryless.

We applied the formalism to membrane proteins fused with the mEos2 fluorescent protein. From the blinking statistics of a monomer and a dimer, we extracted $p$ and $q$, respectively, with $p_0 = p$ for mEos2. The parameters were found to be transferable, which made it possible to determine the oligomerization state of the remaining systems. These results suggest that the counting statistics not only assumes a general form, independent of photophysics, but also that the parameters are not particularly sensitive to the membrane-proximal environment, attesting to the robustness of the method. It will be interesting to explore the blinking statistics in other cellular environments, using other photoswitchable fluorophores, including organic dyes.

Here we used a maximum-likelihood method to extract the model parameters and oligomerization states. Bayesian inference, as a global approach, offers a possible alternative, with suitable priors for the parameters $p_0$, $p$, and $q$ that reflect expectations on the photophysics of the fluorophores and, for the molecule count $m$, on the distribution of oligomerization states.

Finally, we note that our theoretical formulation of counting statistics is entirely general and not limited to fluorophore blinking. It appears that the formalism should apply to the counting of any species for the parameters $p_0(n)$ of the number $n$ of blinking events. Such a transition occurs before eventual bleaching starting from a particular state $k$, we use a generating-function formulation (Bicout and Rubin, 1999; Brown, 2003; Gopich and Szabo, 2003). We define a modified transition matrix $T(z)$ whose elements are $T_{ij}$ except for the $(f, d)$ element, which is multiplied by $z$, $T_{ij}(z) = zT_{ij}(z)$. With $0 < z < 1$, we can think of $T(z)$ as a transition matrix with an additional irreversible process: whenever the system is in state $f$, there is a nonzero probability of “dying,” since $T_{ij}(z) < 1$ for $0 < z < 1$.

For a transition trajectory starting in state $f$ and evolving according to $T(z)$, we define $w_k(z)$ as the probability of reaching the bleached state $N$ instead of dying along the way in state $d$. This probability is the generating function for the transition counts,

$$w_k(z) = \sum_{n=0}^{\infty} p_0(n) z^n$$

This key relation is usually derived using Laplace transforms for specific dynamics (Bicout and Rubin, 1999; Brown, 2003; Gopich and Szabo, 2003). Here, for our transition dynamics, it follows immediately from the expression of the overall probability of going from state $k$ to state $N$ in $M$ transitions according to the modified transition matrix $T(z)$.

**MATERIALS AND METHODS**

In the following, we derive the general expression for the probability $p_m(n)$ of the number $n$ of times fluorophores light up at a particular location with $m + 1$ localized fluorophores, where counting starts after the first light-up event at this location. For a single fluorophore ($m = 0$), $n$ is just the number of blinking events. If multiple fluorophores are localized ($m > 0$), then $n$ can be a combination of other fluorophores lighting up and of blinking events of any of the fluorophores that have already lit up at this position. By lumping together all events after the first lighting up, we account for the fact that, typically, one cannot distinguish between blinking of an already active fluorophore and a new fluorophore lighting up for the first time. We assume that all events of fluorophores lighting up are detected and resolved. We also assume that active fluorophores bleach during the observation time. Moreover, we assume either that we have only one kind of fluorophore or that different fluorophores can be distinguished, for example, by their color. The blinking statistics of localized fluorophores is assumed to be independent. The essence of the approach is to formulate the problem of counting in terms of transitions between microstates, which allows us to ignore the time dependence that would normally be required in kinetic modeling. We will show that the functional form of $p_m(n)$ is independent of the photophysics.

**Single fluorophore**

Consider a single fluorophore with multiple photophysical states $i = 1, 2, \ldots, N$, where the $N$th state is the irreversibly bleached state. We assume that the transitions between the $N$ states are Markovian, that is, probabilistic and independent of the preceding history. Let $T_i = p(i \rightarrow i)$ be the probability that a molecule in state $i$ transitions to state $i$ directly, with $0 \leq T_i \leq 1$ and $\sum_i T_i = 1$. In the conventional kinetic formulation with rate coefficients $K_{ji}$, one would have $T_i = K_{ij} / \sum_k K_{kj}$ for $i \neq j$ and $T_i = 1 - \sum_k K_{ki}$. Our formulation accounts for all such kinetic models and more complex descriptions of the dynamics, possibly with nonexponential waiting times, albeit with Markovian transition probabilities between states. Of importance, we do not explicitly include the inactive state(s) of the fluorophore in our formulation. Instead, we deal with fluorophore activation implicitly by starting the blinking count only after activation of a first fluorophore and lumping the activation of additional localized fluorophores together with the (indistinguishable) blinking events of already active fluorophores.

Now let transitions from state $j = f$ to state $i = f$ correspond to the blinking event of interest, with state $d$ being dark and state $f$ fluorescing. To determine the probability $p_0(n)$ of the number of times $n$ such a transition occurs before eventual bleaching starting from a particular state $k$, we use a generating-function formulation (Bicout and Rubin, 1999; Brown, 2003; Gopich and Szabo, 2003). We define a modified transition matrix $T(z)$ whose elements are $T_{ij}$ except for the $(f, d)$ element, which is multiplied by $z$, $T_{ij}(z) = zT_{ij}(z)$. With $0 \leq z \leq 1$, we can think of $T(z)$ as a transition matrix with an additional irreversible process: whenever the system is in state $f$, there is a nonzero probability of “dying,” since $T_{ij}(z) < 1$ for $0 < z < 1$.

For a transition trajectory starting in state $f$ and evolving according to $T(z)$, we define $w_k(z)$ as the probability of reaching the bleached state $N$ instead of dying along the way in state $d$. This probability is the generating function for the transition counts,

$$w_k(z) = \sum_{n=0}^{\infty} p_0(n) z^n$$

This key relation is usually derived using Laplace transforms for specific dynamics (Bicout and Rubin, 1999; Brown, 2003; Gopich and Szabo, 2003). Here, for our transition dynamics, it follows immediately from the expression of the overall probability of going from state $k$ to state $N$ in $M$ transitions according to the modified transition matrix $T(z)$.

In this path integral (or, more appropriately, path sum) representation of the propagator, evaluated conveniently as the ($N$, $k$) element of the $M$th power of $T(z)$ and then represented as a power series in $z$, the coefficient of $z^k$ is exactly the combined contribution to the overall $k$ to $N$ transition probability for the unmodified dynamics (i.e., for $z = 1$) of all paths in which the transition $d \rightarrow f$ has occurred exactly $n$ times.

By definition, the generating function $w_k(z)$ is identical to the so-called committor (or splitting) probability. We assume that any fluorophore light up eventually bleaches, here by reaching state $N$, which allows us to take the limit of infinitely many transitions, $M \rightarrow \infty$. Then, by the conservation of probability, $w_k(z) = \sum w_k(z) T_{ij}(z)$. With $w_k(z) = 1$ by definition, we thus arrived at the usual expression for the committor in terms of the adjoint of the evolution operator (Onsager, 1938; here the transpose of $T(z)$). Therefore, the vector $w(z)$ of the $N-1$ probabilities $w_1(z), \ldots, w_{N-1}(z)$ of reaching state $N$ without dying in the special state $d$ satisfies

$$w(z) = \tilde{T}^{-1}(z) \mathbf{1}$$

where $\tilde{T}_{ij}(z)$ is an $(N-1) \times (N-1)$ matrix of elements

$$\tilde{T}_{ij}(z) = T_{ij}(z) - \delta_{ij}$$
for $1 \leq i, j \leq N - 1$ (i.e., excluding the bleached state $N_i$), $\mathbf{T}(z)$ is thus the transpose of the first $N - 1$ rows and columns of $\mathbf{T}(z)$ minus the identity matrix, the Kronecker $\delta_{ij}$ being $1$ for $i = j$ and $0$ otherwise. The vector $t$ has elements

$$t_i = -T_{ii}$$

(4)

for $i = 1$ to $N - 1$ and is thus independent of $z$. We note in passing that identical expressions for $w(z)$ are obtained if one takes the infinite-transition limit, evaluating $P(z) = \lim_{m \to \infty} \mathbf{T}^m(z)$ explicitly in a spectral expansion, or by using the fact that $P(z)$ is a projector that satisfies $P(z)\mathbf{T}(z) = \mathbf{T}(z)P(z) = P(z)^2 = P(z)$.

To derive the most general functional form of the generating functions $w_k(z)$, we express the matrix inverse in Eq. 2 in terms of determinants, as $[\ldots]$. We have $\mathbf{T}^{-1} = \text{adj} (\mathbf{T})$, where $\text{adj}(\mathbf{T}) = (-1)^{N-k} \mathbf{M}_k$ with $\mathbf{M}_k$ the $(N-2) \times (N-2)$ matrix constructed by deleting row $i$ and column $j$ in $\mathbf{T}$. From the definition of determinants as sums over all signed permutations of the matrix coefficients, it follows that $w_k(z)$ is a rational function of $z$ in which the denominator is linear in $z$ and the numerator is either constant (independent of $z$) or linear in $z$. Here we exploited the fact that only the $(i, j)$ element of $\mathbf{T}$ depends on $z$. We thus arrive at the most general form of the generating function,

$$w^{(0)}(z) = \frac{p_0 + z(p - p_0)}{1 - z(1 - p)}$$

(5)

where we added a superscript “(0)” to indicate that we have a single fluorophore and took advantage of the fact that the conservation of probability requires $w_k(z = 1) = 1$. In Eq. 5, we averaged over the initial states $k$. After photoactivation, we assume the fluorophore to be in one of the fluorescent states $k$ with probability $p_k$ (with $p_k = 0$ for nonfluorescent states), such that $w^{(0)}(z) = \sum_k p_k w_k(z)$ is a weighted average over the generating functions for the blinking counts starting in state $k$. Changing the probabilities $p_k$ can alter the value of $p_0$ but not the form of $w^{(0)}(z)$. The parameters $p$ and $p_0$ in Eq. 5 are sums of products of the transition probabilities $T_{ij}$ and thus reflect the photophysics of the fluorophore, as illustrated later by specific examples.

The functional form of Eq. 5 of $w^{(0)}(z)$ corresponds to a renewal process (Cao and Silbey, 2008) in two steps, or in one step if $p = p_0$. We note that explicit expressions for $w_k(z)$ can be derived using the Sherman–Morrison formula for matrix inverses. Because, by definition, $0 \leq w_k(z), p_0(n) \leq 1$, the two parameters are themselves probabilities, $0 \leq p, p_0 \leq 1$ (see also Eq. 9 later). As we shall show later, for simple fluorophores with single dark and fluorescent states, we have $p = p_0$ and thus a $z$-independent numerator as a further simplification.

Multiple colocalized fluorophores

When multiple fluorophores are colocalized, an observed blinking event can be caused either by blinking of an already active fluorophore or by a previously inactive fluorophore lighting up for the first time. The generating function of the probability $p_m(n)$ of counting $n$ uncorrelated events for $m + 1$ colocalized fluorophores is thus

$$w^{(m)}(z) = w^{(0)}(z)[q + (1 - q)zw^{(0)}(z)]^m$$

(6)

where the first $w^{(0)}(z)$ accounts for the first fluorophore to light up. The second term accounts for the remaining $m$ fluorophores. In this term, $w^{(0)}(z)$ is thus multiplied by $z$ because lighting up is counted as an event that, we assume, cannot be distinguished from blinking. (Note that in the generating-function formalism, multiplying by $z$ amounts to increasing the count by 1, and multiplying generating functions assumes that counts of the factors—here of the $m + 1$ colocalized fluorophores—are statistically independent.) The initial activation of a fluorophore during the observation time is weighted by the probability $1 - q$, where $q$ is the fraction of fluorophores that do not light up during the observation time, in particular due to incomplete assembly or damage other than eventual photobleaching.

By combining Eqs. 5 and 6, we arrive at the most general form of the generating function. By using the definition of the generating function, $w^{(m)}(z) = \sum_{n=0}^{\infty} p_m(n) z^n$, the binomial theorem $(x + y)^N = \sum_{n=0}^{N} \binom{N}{n} x^{N-n} y^n$, and the geometric series $1/(1 - x) = \sum_{n=0}^{\infty} x^n$ for $|x| < 1$, we obtain the general expression for the probability $p_m(n)$ of $n$ counts, given $m + 1$ colocalized identical fluorophores,

$$p_m(n) = \left\{ \begin{array}{ll} p_0 q^n & \text{if } n = 0 \\ m \sum_{k=0}^{m} p^{m-k} q^n \binom{m}{k} \binom{n}{k} & \text{if } 0 < n \leq m \\ f_m(n) & \text{if } n > m \end{array} \right.$$  

(7)

where $m \choose k = m!/(m-k)!k!$ is the binomial coefficient and

$$f_m(n) = m \sum_{k=0}^{m} \sum_{j=0}^{\infty} \binom{m-k}{j} q^{m-k-j} (1 - q)^j \left( \sum_{p=0}^{n} p^{m-k-j} q^{m-k-j} \right) = m \sum_{j=0}^{\infty} \binom{m}{j} \sum_{k=0}^{m} \binom{m-k}{j} q^{m-k-j} (1 - q)^j \left( \sum_{p=0}^{n} p^{m-k-j} q^{m-k-j} \right)$$

(8)

For $m = 0$ specifically, we obtain

$$p_0(n) = \left\{ \begin{array}{ll} p_0 & \text{if } n = 0 \\ (1 - p_0) p (1 - p)^{n-1} & \text{if } n \geq 1 \end{array} \right.$$  

(9)

In a computer, the probabilities $p_m(n)$ can be conveniently evaluated by recursion:

$$p_{m+1}(n+1) = (1 - p) p_m(n+1) + q p_m(n+1)$$

$$+ [p_0 (1 - q) - q (1 - p)] p_m(n) + (1 - q) (p - p_0) p_m(n - 1)$$

starting from Eq. 9, $p_m(-1) = 0$, and $p_m(0) = p_0 q^n$. This recursion formula was obtained from Eq. 6 by matching the coefficients of $z^n$.

Simple fluorophores

If $p_0 = p$, the count probability simplifies to

$$p_m(n) = \sum_{k=0}^{\min(m,n)} \binom{m}{k} \binom{n}{k} q^{m-k} (1 - q)^k p^{k+1} (1 - p)^{n-k}$$

$$= zF_1 \left[-m,-n;1; \frac{1 - q}{q(1 - p)}; q(1 - p)^n \right]$$

(11)

expressed compactly in terms of a hypergeometric function. For $m = 0, 1$, and $2$ and $p_0(n) = p (1 - p)^n$, we obtain
\[ p_0(n) = p(1-p)^n \]  \hspace{1cm} (12)
\[ p_1(n) = p(1-p)^{n-1} \left[ np(1-q) + q(1-p) \right] \]  \hspace{1cm} (13)
\[ p_2(n) = p(1-p)^{n-2} \left[ n^2 p^2(1-q)^2 + 2a^2(1-p)^2 \right. \]
\[ \left. + np(1-q)(4q - p(1+3q)) \right] / 2 \]  \hspace{1cm} (14)

Note that for simple fluorophores in the limit of \( q \to 0 \) (i.e., all fluorophores are active), we recover the negative binomial distribution derived previously by Lee et al. (2012) using kinetic modeling, albeit with one difference. Because we count the initial light-up of a fluorophore as an event, the distribution \( p_m(n) \) here is shifted to larger \( n \) values by exactly \( m \), that is, \( p_m(n-m) \) for \( q = 0 \) is the negative binomial distribution.

**Mean and variance**

From the \( k \)th derivative of the generating function with respect to \( z \) evaluated at \( z = 1 \), we obtain the factorial moments of the number of counts \( n \) for \( m = 1 \) colocalized fluorophores as

\[ \langle n \rangle = p^{-1} \left[ 1 - p_0 + m(1-q)(1+p-p_0) \right] \]  \hspace{1cm} (15)

reducing to \( \langle n \rangle = p^{-1} [1-p+m(1-q)] \) for the special case of \( p = p_0 \).

For the variance of \( n \), we find

\[ \langle n^2 \rangle - \langle n \rangle^2 = p^{-2} \left[ (1-p_0-p)(1-p_0) + m(1-q) \right] \]
\[ + m(1-q)(1+p-p_0)^2 \]  \hspace{1cm} (16)

which reduces to \( p^{-2} [1-p+m(1-q)(1+q-p)] \) for \( p = p_0 \).

**Simple fluorophore (D ↔ F → B)**

To illustrate how photophysics determines the model parameters \( p \) and \( p_0 \), we first consider the simplest case of a fluorophore with three states \( \{i = 1, 2, 3\} : D \) (dark), \( F \) (fluorescent), and \( B \) (bleached). In this model, bleaching occurs only from the fluorescent state and is irreversible. The nonzero elements of the modified transition matrix are \( T_{12} = 1-p, \ T_{21}(z) = z, \ T_{32} = p, \) and \( T_{33} = 1 \), where \( p \) is the probability of bleaching from the fluorescent state. From Eq. 2, we obtain the generating function for a single active fluorophore \( w(z) = w^{(0)}(z) = p[1 - z(1-p)]^{-1} \), that is, we have the special case of \( p_0 = p \). Accordingly, the blinking statistics for \( m = 1 \) colocalized fluorophores follows \( p_m(n) \) in Eq. 11.

To account for the possibility that bleaching occurs also from the dark state, \( D \to B \), with a probability \( r \), we set \( T_{12}(z) = z(1-r) \) and \( T_{31} = r \). The generating function, \( w(z) = w^{(0)}(z) = u[1 - z(1-u)]^{-1} \), where \( u = p(1-r) + r, \) thus falls again into the simple one-parameter category. However, the interpretation of the single coefficient (now \( u \) instead of \( p! \)) has changed, since \( u \) is a combination of the probabilities \( p \) and \( r \) of bleaching in the fluorescent and dark states, respectively.

**Two fluorescent states (D ↔ F \( _1 \) ↔ F \( _2 \) → B)**

In the case of two fluorescent states in series, the modified transition matrix has nonzero elements \( T_{12} = 1-r, \ T_{21}(z) = z, \ T_{23} = 1-p, \ T_{32} = r, \ T_{43} = p, \) and \( T_{44} = 1 \), where \( r \) is the probability of transitioning from \( F_1 \) to \( F_2 \), and \( p \) is the probability of bleaching from the fluorescent state \( F_2 \). For simplicity, we assume that after photoactivation, we have an equilibrium of \( F_1 \) and \( F_2 \) states. The generating function for the number of blinking events starting from \( F_1 \) or \( F_2 \) according to this equilibrium assumes the general form \( w^{(0)}(z) = \left[ (1-p)w(z) + nw_2(z) \right] / (1+r+p) = [p_0 + z(\rho - p_0)] / [1 - z(1-\rho)] \) with \( \rho = pr / [1-r(1-p)] \) and \( p_0 = \rho(2-p)/(1-p+r) \). In this case, the counting statistics for \( m = 1 \) fluorophores adopts the more complex form of Eq. 7 with \( \rho \) instead of \( p \).

**Two dark states**

We also considered the case of two dark states, \( D_1 \) and \( D_2 \), and one fluorescent state \( F \), a model that was found to describe the photophysics of two popular photoactivatable fluorescent proteins in SMLM, Dendra2 and mEos2 (Lee et al., 2012). For the sake of generality, we allowed all states to interconvert into each other in principle and to photobleach. The resulting transition matrix \( T \) is thus dense. Nonetheless, the generating function \( w^{(0)}(z) \) of the blinking counts starting from the \( F \) state assumes the simple form of Eq. 11 for \( p = p_0 \). However, in this most general case, the coefficients \( p = p_0 \) are relatively complicated sums of products of the transition matrix elements.

**Indistinguishable blinking transitions**

A possible complication arises if different transitions \( d_i \to f_i \) \((i = 1,2,\ldots)\) between distinct dark states \( d_i \) and fluorescent states \( f_i \) result in blinking but cannot be distinguished. To lump together the counts of all \( d_i \to f_i \) transitions, we multiply all corresponding elements in the transition matrix with \( z \), \( T_{i d}(z) = zT_{i d} \), and then determine the generating functions \( w_i(z) \) using Eq. 2. If the transitions \( i \) share either a common fluorescent state \( (f_i = f_j = \ldots) \) or a common dark state \( (d_i = d_j = \ldots) \), all \( z \)-containing elements will be in a row or a column of \( T(z) \), respectively (as, e.g., in the preceding example of two fluorescent states). Following the foregoing derivation and once more invoking the definition of determinants, one finds that the generating function again takes Eq. 5 as its most general form, irrespective of having lumped together counts for different transitions \( d_i \to f_i \). By contrast, if neither the fluorescent nor the dark states are common, the generating functions \( w_i(z) \) for the probability of the number of transitions will still be a rational function of \( z \). However, the order of the \( z \)-polynomials in the numerator and denominator can be higher than linear. Note that if the different transitions \( i \) can be distinguished, then we can use the generating-function approach to calculate the joint probabilities \( p(n_1,n_2,\ldots) \) of seeing \( n \) transitions of type \( i = 1, n_2 \) transitions of type 2, and so on in the same trace. If the respective transition matrix elements are multiplied by \( z_i, \ T_{i d}(z_i,z_2,\ldots) = z_i T_{i d} \), and \( w_i \) is constructed as above, then the coefficient of the \( z^n m^m \) term in the series expansion of \( w_k(z_1,z_2,\ldots) \) is the joint probability.

**From experiment to molecule counts**

The explicit expressions for the count probabilities \( p_m(n) \) in Eqs. 7 and 11 make it possible to use likelihood-based approaches to decide between the simple case of \( p = p_0 \) and the more complex case and infer the unknown parameters \( (p, p_0, q, m) \) from the observed blinking statistics. We define \( c(n) \) as the number of spots at which exactly \( n \) blinking events have been counted, with \( N_{\text{spec}} = \sum c(n) \) the total number of spots analyzed. \( c(n) \) is thus the frequency distribution of an integer number of blinking counts, whose construction does not require binning of a continuous variable. For uncorrelated events and only counting noise, the log-likelihood function is...
\[ L = \sum_{n=0}^{\infty} c(n) \ln p_m(n) \]  

In a maximum-likelihood approach, \( L \) is maximized with respect to the parameters entering \( p_m(n) \). Alternatively, in a Bayesian formulation, we could use priors on the parameters that reflect our expectations on these parameters and use \( L \) to define the posterior.

In the simple case of \( p = p_0 \) for a single fluorophore, \( m = 0 \), maximization of \( L \) with respect to \( p \) results in \( p = 1/(n+1) \). In the general case with \( p \neq p_0 \) and \( m = 0 \), \( L \) is maximal for \( p_0 = c(0)/N_{\text{total}} \) and \( p = (1-p_0)/n \). The maximum-likelihood solution for \( q \), with \( m > 0 \), has to be determined numerically, for example, by Newton–Raphson iteration or bisection. According to the BIC, the log-likelihood \( L \) should increase by at least \( \ln \sum_{n=0}^{\infty} c(n) \) to justify the more complex model, Eq. 7 with \( p \neq p_0 \) over the simple model, Eq. 11.

Unresolved events

If blinking events are fast, not all of them may be resolved. A simple way to account for missed events is to assume that events are resolved with probability \( r \) and missed with probability \( 1-r \). The observed distribution is then

\[ p_{\text{obs},m}(k) = \sum_{n=k}^{\infty} p_m(n) \binom{n}{k} r^k (1-r)^{n-k} \]

The 4th factorial moments of the observed and actual numbers of counts are related by \( \langle n(n-1)\ldots(n-k+1) \rangle_{\text{obs}} = r^k \langle n(n-1)\ldots(n-k+1) \rangle \). In a further extension of this formulation, one could introduce also false positives that arise, for example, from contaminations. If these are not treated properly, a few large \( n \) might have an undue influence on the results.

Our approach is compatible with overlapping blinking cycles. However, if more than one molecule lights up at the same time in a particular spot, this may be detected as only one blinking event, leading to underestimation of \( n \). We expect this scenario to be extremely rare even at higher molecular densities; it can be circumvented by adjusting the experimental activation settings such that only low densities of molecules light up.

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