

## Article

# How Long Should a System Be Observed to Obtain Reliable Concentration Estimates from the Measurement of Fluctuations?

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**ABSTRACT** The interior of cells is a highly fluctuating environment. Fluctuations set limits to the accuracy with which endogenous processes can occur. The physical principles that rule these limits also affect the experimental quantification of biophysical parameters in situ. The characterization of fluctuations, on the other hand, provides a way to quantify biophysical parameters. But as with any random process, enough data has to be collected to achieve a reliable quantitative description. In this article we study the accuracy with which intracellular concentrations can be estimated using fluorescence correlation spectroscopy. We show that, when the observed molecules interact with immobile species or experience other restrictions to their movement, the hypotheses commonly used to estimate concentrations are no longer valid. The interactions with immobile sites reduce the fluorescence variance by a finite amount. The time that is necessary to obtain an accurate concentration estimate, on the other hand, is hundreds of times larger than the slowest correlation time and is much larger when the sites move slowly than when they are immobile. Our analysis is applicable to other related techniques and it also sheds light on the way in which effector concentrations are read by target molecules in cells.

## INTRODUCTION

The use of fluorescently marked molecules and the development of new optical techniques have given the opportunity to observe intracellular processes at work with great resolution. The experiments allow a direct visualization of the large fluctuations that affect these processes. These fluctuations not only impact directly on the efficiency of the mechanisms that underlie cell function but also hinder the quantification of biophysical parameters from experiments performed in situ. There are several optical techniques, however, that exploit fluctuations to extract quantitative information, particularly on transport rates and concentrations. Fluorescence correlation spectroscopy (FCS) and its variants (1–10) are among these techniques. In all of them the fluorescence in a given (small) volume is observed for a long time. Transport rate or concentration estimates are inferred based on a statistical analysis of the fluorescence fluctuations. Reliable estimates are then derived if the system is in a stationary state and is observed for a long enough time.

The techniques are subject to two main sources of fluctuations: those in the number of fluorescent molecules in the observation volume and those in the number of detected photons per sampling time. The former are the basis of what is the signal for the experiments. The latter correspond to noise because the number of counted photons at different times is uncorrelated (3). Here we are interested in molecule number fluctuations, and how they set bounds on the time

during which the system must be observed to derive concentration estimates with a given accuracy. The limits imposed by physics upon the precision of these estimates also limits the precision of the endogenous intracellular mechanisms used to sense concentrations to perform actions. Thus, our study not only has implications for the experimental quantification of biophysical parameters but also sheds light on the way in which effector concentrations are read by target molecules in cells (11–13).

In FCS the fluorescence,  $F(t)$ , in an observation volume,  $V_{\text{obs}}$ , emitted by the fluorescently tagged molecules of interest, is monitored for a time,  $T_{\text{obs}}$  (1,10).  $V_{\text{obs}} \lesssim 1$  fL and the key concentrations are approximately uniform and in equilibrium inside it. The autocorrelation function (ACF) of the fluorescence fluctuations,  $\delta F \equiv F(t) - \langle F \rangle$ , is computed as  $G(\tau) = \langle \delta F(t) \delta F(t + \tau) \rangle / \langle F \rangle^2$  (3). The total weight,  $G_o \equiv G(\tau = 0)$ , of the ACF, in principle, is given by

$$G_o = \frac{\text{var}(F)}{\langle F \rangle^2} = \frac{\langle (F - \langle F \rangle)^2 \rangle}{\langle F \rangle^2}, \quad (1)$$

provided that the variance and the mean are estimated correctly from the experiment. As we have already mentioned,  $\text{var}(F)$  depends on fluctuations in the number of fluorescent molecules in  $V_{\text{obs}}$  and in the number of detected photons, but we will focus on the former. We discuss later how photon-counting fluctuations affect our results. For now, we will proceed as if they did not exist. In such a case, if the fluorescent molecules obey Poisson statistics, the total weight of the ACF satisfies

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$$G_o = \frac{1}{\langle N^l \rangle}, \quad (2)$$

with  $\langle N^l \rangle$  as the mean number of fluorescent molecules in  $V_{\text{obs}}$ . Using Eq. 2,  $\langle N^l \rangle$  is typically estimated from  $G_o$  in experiments. The assumptions that lead to this equation do not hold when the fluorescent molecules react with immobile sites, in which case, as we show in this article, there is a different relationship between  $\langle N^l \rangle$  and  $G_o$ . Regarding time, the ACF is often the sum of components,  $G_i(\tau) = G_{o_i} f_i(\tau)$ , each one with individual weight,  $G_{o_i}$ , and associated to a branch of eigenvalues of a linear dynamical system (3). In the case of a single freely diffusing species there is only one component, ( $i = 1$ ), of the form

$$G_i(\tau) = G_{o_i} \left(1 + \frac{\tau}{\tau_i}\right)^{-1} \left(1 + w^{-2} \frac{\tau}{\tau_i}\right)^{-1/2}, \quad (3)$$

where

$$\tau_i = \frac{w_r^2}{4D_i}$$

with  $D_i$  as the diffusion coefficient and  $w_r$  and  $w_z \equiv ww_r$  the width of  $V_{\text{obs}}$  on the focal plane and along the optical axis, respectively. For simplicity, in what follows, we will assume  $w = 1$ .

In cells, most species diffuse and bind/unbind to sites, in which case there is not an algebraic expression for the ACF. There are two limits in which its components recover the form of Eq. 3 (14), i.e., the fast diffusion and the fast reaction limits, where the correlation times are determined, respectively, by the free diffusion coefficients of the observed species and by effective coefficients that depend on concentrations and reaction rates (15,16) (see [Materials and Methods](#)). In this article we focus on systems in which the marked molecules interact with immobile or slowly moving binding sites. We study the systems in the fast reaction limit because there is an analytic expression for the ACF with reactions playing a role (17). As we discuss later, our main results still apply outside this limit. In particular, we find very different results depending on whether the binding sites are immobile or slowly moving.

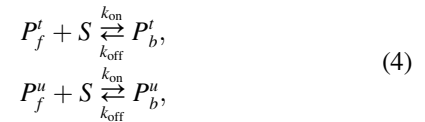
More specifically, we determine that the interaction with immobile sites introduces correlations that reduce the variance of the observed molecules number with respect to the noninteracting case, a feature that alters the relationship between experimentally available parameters and concentrations. A smaller variance implies smaller errors in the concentration estimates. The time during which the system must be probed to obtain the estimates with a given accuracy in this case depends on a relatively fast timescale. When the binding sites move slowly, the observation time can be very long and much larger than the time it takes to infer transport rates in FCS with the same accuracy. The very slow trans-

port timescales, however, can go undetected in the experiment, in which case the idea of transient concentrations is inferred. This transient detection also occurs in signaling whereby the rapid sensing of concentration changes can expand the dynamic range of the detection process in the presence of saturating ligand concentrations (18).

## MATERIALS AND METHODS

### The systems

For most of the computations, we consider a reaction-diffusion system (14–16) composed of particles,  $P_f$ , that diffuse with free coefficient,  $D_f$ , and react with binding sites,  $S$ . We assume that the particles can be fluorescent,  $P_f^l$ , (tagged) or nonfluorescent,  $P_f^u$  (untagged), but are otherwise identical, and that their interconversion does not occur during the duration of the experiment. We assume that the reaction with the sites, which is given by



where the rates of binding and unbinding define the dissociation constant,  $K_D \equiv k_{\text{off}}/k_{\text{on}}$ , which does not alter the photophysical properties of the particles. Thus, we have a system with five species:  $P_f^l$ ,  $P_b^l$ ,  $P_f^u$ ,  $P_b^u$ , and  $S$ . We assume that the binding sites belong to molecules that are much larger than the particles so that both  $P_b^{l,u}$  and  $S$  diffuse with  $D_S \ll D_f$ . The equilibrium concentrations of the various species,  $[P_f^l]$ ,  $[P_b^l]$ ,  $[P_f^u]$ ,  $[P_b^u]$ , and  $[S]$ , satisfy

$$[P_f^{l,u}][S] = K_D [P_b^{l,u}],$$

$$[P_{f,b}^l] = f_t \left( [P_{f,b}^l] + [P_{f,b}^u] \right),$$

with  $f_t$  the fraction of tagged particles, and  $[P_f^l] + [P_f^u] + [P_b^l] + [P_b^u] = [P]_T$  and  $[S] + [P_b^l] + [P_b^u] = [S]_T$  are the constants. To gain insight into some properties of the reaction-diffusion system we also perform calculations using a simpler system with two noninteracting equally fluorescent species,  $f$  and  $S$ , that diffuse, respectively, with coefficients  $D_f$  and  $D_S$ .

### Autocorrelation function: analytic calculations

For the reaction-diffusion system introduced before, the fluorescence collected by the microscope at time  $t$  is

$$F(t) = \Delta t \int d^3\vec{r} I(\vec{r}) q \left( c_f^l(\vec{r}, t) + c_b^l(\vec{r}, t) \right)$$

(3), with  $\Delta t$  as the sample time,  $I(\vec{r})$  as the distribution of the excitation light in the sample,  $q$  as the product of the absorption cross section, the fluorescence quantum yield, and the efficiency of the fluorophore (which we assume yields the same result for  $P_f^l$  and  $P_b^l$ ), and  $c_f^l$ ,  $c_b^l$  as the number density of fluorescent molecules in their free and bound forms. The latter include particle number fluctuations. For example,  $c_f^l$  is given by

$$c_f^l = \sum_{i_f} \delta(\vec{r} - \vec{r}_{i_f}(t))$$

with the sum running over the free fluorescent particles and  $\vec{r}_{i_f}(t)$  is the location of each of them at time  $t$ . As we explain later, to obtain an

analytic expression of the ACF we actually calculate the difference,  $\delta c_f^t \equiv c_f^t(\vec{r}, t) - [P_f^t]$ , with respect to the equilibrium solution,  $[P_f^t]$  and, simultaneously, the differences for all five species of the system, as the solution of a linear reaction-diffusion system. It is implicit in this calculation that the mean number of molecules of each species in the observation volume is proportional to the corresponding equilibrium concentration (e.g.,  $\langle N_f^t \rangle = V_{\text{obs}}[P_f^t]$ ). The autocorrelation function of the fluorescence fluctuations (ACF) is defined and computed as (3)

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} = T_{\text{obs}} \frac{\int_0^{T_{\text{obs}}} \delta F(t) \delta F(t + \tau) dt}{\left( \int_0^{T_{\text{obs}}} F(t) dt \right)^2}, \quad (5)$$

where  $\delta F(t) = F(t) - \langle F \rangle$  is the deviation of the fluorescence from its mean,  $\langle F \rangle$ ; and  $T_{\text{obs}}$  is the observation time. The observation volume is

$$V_{\text{obs}} = \int d^3 \vec{r} I(\vec{r}).$$

To derive an analytic expression for  $G(\tau)$ , we follow Krichevsky and Bonnet (3). Namely, we solve the (linearized) equations of the reaction-diffusion system for the deviations of the concentrations of the five species with respect to the equilibrium solution,  $[P_f^t]$ ,  $[P_b^t]$ ,  $[P_f^u]$ ,  $[P_b^u]$ , and  $[S]$ , and compute

$$\delta F(t) = \Delta t \int d^3 \vec{r} I(\vec{r}) q \sum_{if} \left( \delta c_f^t(\vec{r}, t) + \delta c_b^t(\vec{r}, t) \right)$$

using the solution,  $\delta c_f^t$ ,  $\delta c_b^t$ , of the linear system. This solution can be written in Fourier space in terms of the (branches of) eigenvalues and eigenvectors of the linear system and the ACF becomes

$$G(\tau) = \frac{(2\pi)^{-3}}{q^2 \left( [P_f^t] + [P_b^t] \right)^2} \times \int d^3 \xi \widehat{I}(\vec{\xi}) \sum_{j,l} q_j q_l \times \sum_s X_{l,s} \exp(\lambda_s \tau) \sum_k (X^{-1})_{s,k} \sigma_{j,k}^2, \quad (6)$$

where the subscripts  $j, l$  label the five species of the reaction-diffusion system; the index,  $(s)$ , labels the eigenvalues;  $\widehat{I}(\vec{\xi})$  is the Fourier transform of  $I(\vec{r})$ ;  $q_i$  is the fluorescence efficiency of the  $i$ th species (it is either zero or  $q$ );  $X$  is the matrix of eigenvectors, and  $X^{-1}$  is its inverse;  $\lambda_s$  is the  $s$ th eigenvalue; and  $\sigma^2$  is the matrix of initial correlations between the species. Both in Krichevsky and Bonnet (3) and here, it is assumed that the initial correlations are spatially short-ranged,  $\langle \delta c_i(\vec{r}, 0) \delta c_j(\vec{r}', 0) \rangle \propto \delta(\vec{r} - \vec{r}')$ . Under this assumption, in the fast reaction limit (which holds when reactions occur on a timescale,  $\tau_r$  (which is faster than that of (free) diffusion across the observation volume,  $\tau_f$  (14)), the ACF of the reaction-diffusion system is the sum of three diffusive components that, considering a Gaussian illumination profile, can be written as (16) (for a detailed calculation, see the [Supporting Material](#))

$$G(\tau) = \frac{G_{os}}{\left(1 + \frac{\tau}{\tau_S}\right) \sqrt{1 + w^2 \frac{\tau}{\tau_S}}} + \frac{G_{ocoll}}{\left(1 + \frac{\tau}{\tau_{coll}}\right) \sqrt{1 + w^2 \frac{\tau}{\tau_{coll}}}} + \frac{G_{osm}}{\left(1 + \frac{\tau}{\tau_{sm}}\right) \sqrt{1 + w^2 \frac{\tau}{\tau_{sm}}}}, \quad (7)$$

$$\begin{aligned} \tau_S &= \frac{w_r^2}{4D_S}, \\ \tau_{coll} &= \frac{w_r^2 \left(1 + \frac{[S]^2}{K_D [S]_T}\right)}{4 \left(D_f + \frac{[S]^2}{K_D [S]_T} D_S\right)}, \\ \tau_{sm} &= \frac{w_r^2 \left(1 + \frac{[S]}{K_D}\right)}{4 \left(D_f + \frac{[S]}{K_D} D_S\right)}, \end{aligned} \quad (8)$$

where  $w = w_z/w_r$  is the ratio of the beam waist along the direction of propagation of the light,  $w_z$ , and along the perpendicular direction,  $w_r$ . In this limit, the weights of the components associated to the two branches of eigenvalues not included in Eq. 7 are negligible. Using

$$\langle \delta c_i(\vec{r}, 0) \delta c_j(\vec{r}', 0) \rangle = \langle \delta N_i \delta N_j \rangle \delta(\vec{r} - \vec{r}') / V_{\text{obs}},$$

we obtain general expressions for the weights of each component of the ACF as functions of the variances and covariances of the random variables of the problem. Choosing subsequently different variances and covariances we extend the calculation beyond the usual assumption of Poisson (uncorrelated) statistics of Krichevsky and Bonnet (3). More specifically, for the weights and characteristic times of each component, we obtain

$$\begin{aligned} G_{os} &= \frac{1}{\langle N^t \rangle^2} \frac{[P_f^t]}{K_D + [P_f^t]_T} \gamma_1, \\ G_{ocoll} &= \frac{1}{\langle N^t \rangle^2} \frac{[P_f^t]}{K_D + [P_f^t]_T} \frac{K_D \gamma_2 - [P_f^t]_T \gamma_3}{[P_f^t]_T}, \\ G_{osm} &= \frac{1}{\langle N^t \rangle^2} \frac{[P_f^u] \gamma_4 - [P_f^t] \gamma_5}{[P_f^t]_T}, \end{aligned} \quad (9)$$

where  $[P_f]_T = [P_f^t] + [P_f^u]$  is the (equilibrium) total free particle concentrations,  $\langle N^t \rangle = V_{\text{obs}}([P_f^t] + [P_b^t])$  is the mean number of fluorescent molecules in  $V_{\text{obs}}$ , and the correlation factors are given by

$$\begin{aligned} \gamma_1 &= \langle \delta N_b^{t2} \rangle + \langle \delta N_f^t \delta N_b^t \rangle + \langle \delta N_f^t \delta N_S \rangle + \langle \delta N_f^t \delta N_b^u \rangle \\ &\quad + \langle \delta N_b^t \delta N_b^u \rangle + \langle \delta N_b^t \delta N_S \rangle, \\ \gamma_2 &= \langle \delta N_f^{t2} \rangle + \langle \delta N_b^{t2} \rangle + 2 \langle \delta N_f^t \delta N_b^t \rangle + \langle \delta N_f^t \delta N_f^u \rangle \\ &\quad + \langle \delta N_f^u \delta N_b^t \rangle + \langle \delta N_f^t \delta N_b^u \rangle + \langle \delta N_b^t \delta N_b^u \rangle, \\ \gamma_3 &= \langle \delta N_f^t \delta N_S \rangle + \langle \delta N_b^t \delta N_S \rangle - \langle \delta N_f^{t2} \rangle - \langle \delta N_f^t \delta N_b^t \rangle \\ &\quad - \langle \delta N_f^t \delta N_f^u \rangle - \langle \delta N_f^u \delta N_b^t \rangle, \\ \gamma_4 &= \langle \delta N_f^{t2} \rangle + \langle \delta N_b^{t2} \rangle + 2 \langle \delta N_f^t \delta N_b^t \rangle, \\ \gamma_5 &= \langle \delta N_f^t \delta N_f^u \rangle + \langle \delta N_f^u \delta N_b^t \rangle + \langle \delta N_f^t \delta N_b^u \rangle + \langle \delta N_b^t \delta N_b^u \rangle, \end{aligned} \quad (10)$$

where  $N_f^t$ ,  $N_b^t$ ,  $N_f^u$ ,  $N_b^u$ , and  $N_S$  are the five random variables of the problem, namely, the number of free and bound particles, both tagged and untagged, and of binding sites in  $V_{\text{obs}}$ . We see from the expressions in Eq. 9 that, in the fast reaction limit, the ACF has three characteristic times (14,16): one associated to the free diffusion of the binding sites,  $D_S$ , and

the other two to the collective and single molecule effective diffusion coefficients,

$$D_{\text{coll}} = \left( D_f + \frac{[S]^2}{K_D[S]_T} D_S \right) / \left( 1 + \frac{[S]^2}{(K_D[S]_T)} \right)$$

and

$$D_{sm} = \left( D_f + \frac{[S]}{K_D} D_S \right) / \left( 1 + \frac{[S]}{K_D} \right),$$

as studied in Pando et al. (15). As previously shown in Ipiña and Dawson (14) and Sigaut et al. (16),  $D_S < D_f$  implies that  $\tau_S \geq \tau_{sm} \geq \tau_{\text{coll}} \geq \tau_f = w_f^2/4D_f$ . Under the usual assumptions that the correlations are spatially short-ranged and that  $N_f^t, N_b^t, N_S, N_f^t$  and  $N_b^t$  are not correlated between themselves and obey Poisson statistics, i.e.,

$$\langle \delta c_i(\vec{r}, 0) \delta c_j(\vec{r}', 0) \rangle = \langle c_i \rangle \delta_{ij} \delta(\vec{r} - \vec{r}')$$

(3), the expressions in Eq. 10 lead to the weights derived in (16)

$$\widehat{G}_{o_{\text{coll}}} = f_i \left( \langle N_f^t \rangle + \frac{[S]}{[S]_T} \langle N_b^t \rangle \right); \quad (11)$$

$$\widehat{G}_{o_{sm}} = (1 - f_i) \langle N^t \rangle,$$

$$\widehat{G}_{o_S} = \frac{[P_b^t]}{[S]_T} \langle N_b^t \rangle, \quad (12)$$

where we have introduced the notation  $\widehat{G}_{o_i} \equiv \langle N^t \rangle^2 G_{o_i}$ . In the Results, we show the weights that are obtained when the binding sites are immobile, a property that introduces correlations between some of the stochastic variables and invalidates the assumption of uncorrelated Poisson statistics.

In the case of the simpler system with two noninteracting equally fluorescent species that diffuse with  $D_f$  and  $D_S \neq 0$ , the ACF is the sum of two terms of the form of Eq. 3 with  $D_1 = D_f$ ,  $D_2 = D_S$ , and  $\widehat{G}_{o_2} = \langle N_S^t \rangle$  with  $N_f^t$  and  $N_S$  the number of fluorescent molecules of the  $f$  and the  $S$  species, respectively, and  $N^t = N_f^t + N_S^t$ . In this case, if  $D_S = 0$ , all fluorescence fluctuations are due to the  $f$  particles diffusion and the ACF has one component with correlation time,  $\tau_f = w_f^2/(4D_f)$ . It must be noted that the ACF of the reaction-diffusion system reduces to that of the system with two noninteracting species in the fast diffusion limit (14).

## ACF weights estimates and their precision

For any system, both the individual,  $G_{o_i}$ , and the total

$$G_o = \sum_i G_{o_i},$$

weights of the ACF, depend on the mean, the variance, and the covariances of the random variables of the problem (the number of molecules in  $V_{\text{obs}}$ ). In an actual experiment, the means, variances, and covariances are estimated from the observations. Thus, the computation of the weights is done in terms of these estimators. Let us call

$$\tilde{G}_o \equiv \frac{s^2}{(\overline{N^t})^2}, \quad (13)$$

the estimator of the total weight with

$$\overline{N^t} = \frac{\sum_{\ell} N_{\ell}^t}{n}; \quad (14)$$

$$s^2 = \frac{\sum_{\ell} (N_{\ell}^t - \overline{N^t})^2}{n-1},$$

the estimators of  $\langle N^t \rangle$  and  $\text{var}(N^t)$ , respectively, where  $N_{\ell}^t$  is the number of fluorescent molecules in  $V_{\text{obs}}$  at time  $t = \ell \Delta t$  and  $n = T_{\text{obs}}/\Delta t$  (i.e., the ratio of the observation to the sampling time of the experiment) is the sample size.  $\overline{N^t}$  and  $s^2$  are unbiased estimators so that their expected values are  $\langle N^t \rangle$  and  $\text{var}(N^t)$ , respectively. The estimators are random variables. Their variance as a function of the sample size,  $n$ , provides information on how different the estimators can be with respect to their expected values after a certain observation time.

We now calculate  $\text{var}(\overline{N^t})$  and  $\text{var}(s^2)$  for the case of a single fluorescent species that diffuses with coefficient,  $D$ . In this case there is a single correlation time,

$$\tau_{\text{corr}} = \frac{w_f^2}{4D}.$$

In any experiment, it is  $\Delta t \ll \tau_{\text{corr}}$ , so that the values that  $N^t$  takes along time,  $\{N_{\ell}^t\}$ , are not independent. Given that

$$\begin{aligned} \text{var}(\overline{N^t}) &= \text{var} \left( \frac{1}{n} \sum_{\ell=1}^n N_{\ell}^t \right) \\ &= \frac{1}{n^2} \sum_{\ell, k=1}^n \langle (N_{\ell}^t - \langle N^t \rangle) (N_k^t - \langle N^t \rangle) \rangle, \end{aligned} \quad (15)$$

we exploit the behavior of the ACF, which in this case satisfies

$$\begin{aligned} \langle N^t \rangle^2 G(\tau) &= \langle (N^t(t) - \langle N^t \rangle) (N^t(t + \tau) - \langle N^t \rangle) \rangle \\ &= \frac{\text{var}(N^t)}{\left( 1 + \frac{\tau}{\tau_{\text{corr}}} \right)^{3/2}} \end{aligned}$$

to approximate

$$\langle (N_{\ell}^t - \langle N^t \rangle) (N_k^t - \langle N^t \rangle) \rangle,$$

by

$$\langle (N_{\ell}^t - \langle N^t \rangle)^2 \rangle = \text{var}(N^t) \text{ if } \Delta t(\ell - k) \geq \tau_{\text{corr}}$$

and 0, otherwise. In this way, we obtain

$$\text{var}(\overline{N^t}) \approx \frac{\text{var}(N^t)}{n} (1 + (n-1)\rho) \quad (16)$$

with

$$\rho = 2 \frac{(n+1)\tau}{(n-1)T_{\text{obs}}}.$$

We obtain a similar result if we use a set of uncorrelated data out of the observations, namely, the sequence of  $n_u \sim T_{\text{obs}}/\tau_{\text{corr}}$  values observed at times  $t_j = j\tau_{\text{corr}}$ . In such a case, we obtain

$$\text{var}(\overline{N^t}) = \frac{\text{var}(N^t)}{n_u} \sim \frac{T_{\text{obs}}}{\tau_{\text{corr}}} \text{var}(N^t)$$

as before. In this uncorrelated case, it is

$$\text{var}(s^2) = \frac{2(\text{var}(N^t))^2}{n_u - 1}.$$

Proceeding similarly with  $s^2$  as with  $\bar{N}^t$ , we obtain

$$\text{var}(s^2) \approx \frac{2(\text{var}(N^t))^2}{n - 1} (1 + (n - 1)\rho), \quad (17)$$

with  $\rho$  as before. The estimates from Eqs. 16 and 17 also apply to each fluorescent species of a multispecies system in the absence of interactions. As shown in the Results, they can also be used with proper correlation times in the case of interacting species.

For systems with several equally fluorescent species with numbers of molecules in  $V_{\text{obs}}$ ,  $N_i^t$ , that are not correlated with those of the other fluorescent species, the total weight and its estimate are given by

$$G_o = \frac{\sum_i \text{var}(N_i^t)}{\langle \sum_i N_i^t \rangle^2}; \quad (18)$$

$$\tilde{G}_o = \frac{\sum_i s_i^2}{\bar{N}^2},$$

where

$$N^t = \sum_i N_i^t$$

and  $\bar{N}_i^t$  and  $s_i^2$  are the estimates of  $\langle N_i^t \rangle$  and  $\text{var}(N_i^t)$ , respectively. Making the identification  $(\Delta \Xi)^2 = \text{var}(\Delta \Xi)$  between the error,  $\Delta \Xi$ , and the variance,  $\text{var} \Delta \Xi$ , for  $\Xi = \bar{N}_i^t, s_i^2$ , we obtain

$$\begin{aligned} \left( \frac{\Delta \tilde{G}_o}{\langle \tilde{G}_o \rangle} \right)^2 &= \frac{1}{\langle \tilde{G}_o \rangle^2} \sum_j \left( \left( \frac{\partial \tilde{G}_o}{\partial s_j^2} \right)^2 \text{var}(s_j^2) + \left( \frac{\partial \tilde{G}_o}{\partial \bar{N}_j^t} \right)^2 \text{var}(N_j^t) \right) = \frac{\sum_i \text{var}(s_i^2)}{\langle \sum_i s_i^2 \rangle^2} + \frac{4 \sum_i \text{var}(\bar{N}_i^t)}{\langle \sum_i \bar{N}_i^t \rangle^2} \\ &= \frac{\sum_i \text{var}(s_i^2)}{(\sum_i \text{var}(N_i^t))^2} + \frac{4 \sum_i \text{var}(\bar{N}_i^t)}{\langle \sum_i N_i^t \rangle^2}. \end{aligned} \quad (19)$$

## Numerical simulations

In order to check our analytic calculations, we perform a series of stochastic numerical simulations using a Gillespie-like algorithm (19), as described in Ipiña and Dawson (14). To compute  $F(t)$ , we weigh the contribution of each particle using a Gaussian profile (mimicking a confocal microscope) or counting all those inside a cube with the same weight. We perform simulations both for the reaction-diffusion and for the simpler system with two noninteracting species using the system parameters listed in Table 1. No-flux boundary conditions were considered. In the case of the reaction-diffusion system, the diffusion coefficients and the dissociation constant correspond to those derived from an analysis (17) of FCS experiments performed in embryos of *Drosophila melanogaster* to estimate the diffusion coefficient of the protein Bicoid (20).

## RESULTS

In this section, we first compare the correlation times and weights of the ACF for the reaction-diffusion system with immobile and with mobile binding sites. We then analyze how a finite observation volume affects the weights of the ACF. We then study the accuracy of the estimates of the ACF weights as a function of the observation time for a system with freely diffusing molecules and for the reaction-diffusion system. To perform the analyses, we apply the calculations presented in Materials and Methods to the two systems under study.

### ACF for a reaction-diffusion system with immobile binding sites in the case of a small observation volume

When the binding sites are immobile (i.e.,  $D_S = 0$ ), their number inside  $V_{\text{obs}}$  is fixed, i.e.,  $N_{S_T} = N_S + N_b^i + N_b^t$  is

**TABLE 1** Simulation parameters used to make the figures of this article

Parameter	Fig. 1 a	Fig. 1 b	Fig. 2 a	Fig. 2 b	Fig. 3
$D_S$	[0–10] $\mu\text{m}^2 \text{s}^{-1}$	10 $\mu\text{m}^2 \text{s}^{-1}$	0.005 $\mu\text{m}^2 \text{s}^{-1}$	0	10 $\mu\text{m}^2 \text{s}^{-1}$
$D_f$	19 $\mu\text{m}^2 \text{s}^{-1}$	19 $\mu\text{m}^2 \text{s}^{-1}$	500 $\mu\text{m}^2 \text{s}^{-1}$	19 $\mu\text{m}^2 \text{s}^{-1}$	19 $\mu\text{m}^2 \text{s}^{-1}$
$k_{\text{off}}$	400 $\text{s}^{-1}$	400 $\text{s}^{-1}$	—	400 $\text{s}^{-1}$	0.1 $\text{s}^{-1}$
$K_D$	0.2496 $\mu\text{M}$	0.2496 $\mu\text{M}$	—	0.2496 $\mu\text{M}$	0.192 nM
[S]	2.87 $\mu\text{M}$	2.87 $\mu\text{M}$	—	2.87 $\mu\text{M}$	2.21 nM
$[P_f^i]$	5.90 $\mu\text{M}$	5.90 $\mu\text{M}$	0.01 $\mu\text{M}$	5.90 $\mu\text{M}$	4.54 nM
$[P_b^i]$	68.29 $\mu\text{M}$	68.29 $\mu\text{M}$	0.01 $\mu\text{M}$	68.29 $\mu\text{M}$	52.53 nM
$[P_f^u]$	1.78 $\mu\text{M}$	1.78 $\mu\text{M}$	—	1.78 $\mu\text{M}$	1.37 nM
$[P_b^t]$	20.02 $\mu\text{M}$	20.02 $\mu\text{M}$	—	20.02 $\mu\text{M}$	15.40 nM
$T_{\text{obs}}$	100 s	100 s	[0.03–11–1000]s	[0.04–0.64–10–82]s	100 s
$I(\vec{r})$	Gaussian	Gaussian	Gaussian	Gaussian	Cubic
$V_{\text{obs}}$	0.15 $\mu\text{m}^3$	0.36 $\mu\text{m}^3$	0.068 $\mu\text{m}^3$	0.15 $\mu\text{m}^3$	0.064 $\mu\text{m}^3$
$V_T$	27 $\mu\text{m}^3$	[8–64] $\mu\text{m}^3$	27 $\mu\text{m}^3$	27 $\mu\text{m}^3$	27 $\mu\text{m}^3$

constant. Thus,  $\delta N_b^t + \delta N_S + \delta N_b^u = 0$ , so that  $N_b^t$ ,  $N_S$ , and  $N_b^u$  are correlated (they obey multinomial statistics) and satisfy

$$\langle \delta N_i^2 \rangle = \langle N_i \rangle \left( 1 - \frac{\langle N_i \rangle}{N_{ST}} \right)$$

and

$$\langle \delta N_i \delta N_j \rangle = -\langle N_i \rangle \frac{\langle N_j \rangle}{N_{ST}},$$

for  $i \neq j$ . Assuming that there are no other correlations and that  $N_f^t$  and  $N_b^t$  obey Poisson statistics, using Eq. 10 we obtain  $\gamma_i = 0$ . The weight associated to  $D_S$  becomes  $G_{oS} = 0$  for  $D_S = 0$ . The other factors,  $\gamma_i$ , remain as in the usual (Poissonian) case, so that  $G_{o\text{coll}}$  and  $G_{o\text{sm}}$  are still given by Eq. 11. Thus, the total weight,

$$G_o = G_{o\text{coll}} + G_{o\text{sm}} + G_{oS} = \frac{\widehat{G}_{o\text{coll}} + \widehat{G}_{o\text{sm}} + \widehat{G}_{oS}}{\langle N^t \rangle^2}, \quad (20)$$

equals  $(\text{var}(N_f^t) + \text{var}(N_b^t))/\langle N^t \rangle^2$  but, because

$$\text{var}(N_b^t) = \langle N_b^t \rangle \left( 1 - \frac{\langle N_b^t \rangle}{N_{ST}} \right) = \langle N_b^t \rangle \left( 1 - \frac{P_b^t}{[S]_T} \right),$$

then

$$G_o \neq 1/\langle N^t \rangle.$$

If  $D_S \neq 0$  and  $V_{\text{obs}} \ll V_T$  with  $V_T$  the total accessible volume of the system, then  $N_S$ ,  $N_b^t$ , and  $N_b^u$  are Poisson (instead of multinomial) distributed. In such a case, Eq. 11 still holds but  $\widehat{G}_{oS}$  is given by Eq. 12, which is independent of  $D_S$ . Thus, the total weights  $G_o$  for  $D_S = 0$  and  $D_S \neq 0$  differ by a finite amount because of the correlations that the reaction introduces. This is illustrated in Fig. 1 a, where we show the ACF of two numerically generated fluorescence time-series (*symbols*) and the corresponding analytic expressions (*lines*). The parameters are the same in both simulations (see Table 1) except for  $D_S$ , which is 0 (*squares* and *solid*

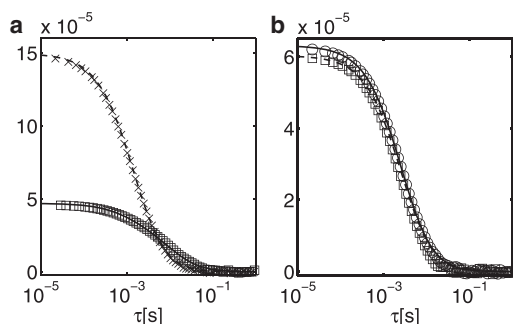


FIGURE 1 (a) ACF,  $G(\tau)$ , of a system of particles that diffuse and react with immobile (*squares*) or mobile (*crosses*) sites. The curves are the theoretical predictions for  $D_S \neq 0$  (*dashed*) and  $D_S = 0$  (*solid*). (b) Similar to panel a, but for  $D_S \neq 0$  and  $V_{\text{obs}} = 0.36 \mu\text{m}^3$ ,  $V_T = 8 \mu\text{m}^3$  (*circles*).

*curve*) or  $D_S = 10 \mu\text{m}^2/\text{s}$  (*crosses* and *dashed curve*).  $G_o$  is different in each case, although  $\langle N^t \rangle$  is the same. Thus, a blind fit of the data assuming Poisson statistics in both cases would result in two very different concentrations.

### ACF for cases with comparable observation and total available volumes

When  $V_{\text{obs}}$  is large compared to the total volume over which the molecules diffuse,  $V_T$ , the Poisson statistics does not hold even if  $D_S \neq 0$ . Namely, the total number of fluorescent particles in  $V_{\text{obs}}$ ,  $N^t = N_f^t + N_b^t$ , is correlated with the corresponding number outside  $V_{\text{obs}}$ . As a result of this, the variance of  $N^t$  and, hence the total weight,  $G_o$ , are multiplied by the factor

$$\left( 1 - \frac{V_{\text{obs}}}{V_T} \right)$$

with respect to the  $V_{\text{obs}} \ll V_T$  case. This is illustrated in Fig. 1 b, where the parameters are such that  $1 - V_{\text{obs}}/V_T$  varies from 0.96 to 0.99 between the two portrayed simulations. This difference should result, according to the theory, in an  $\sim 4\%$  variation of the total weight, which is what we obtain with the simulations.

### Observation time that is needed to obtain estimates of the ACF weights with a given accuracy

#### Freely diffusing particles with disparate diffusion coefficients

As described before and illustrated in Fig. 1 a, the difference between the weights,  $G_{oS}$ , for  $D_S = 0$  and for  $D_S \neq 0$ , is finite and independent of  $D_S$ . Thus, the  $D_S = 0$  case corresponds to a singular limit. We now analyze if, for  $D_S$  small enough, there is a range of  $T_{\text{obs}}$  values for which the ACF can be approximated by an expression with  $G_{oS} = 0$ . To this end, we first consider the simpler system with two types of equally fluorescent molecules ( $f$  and  $S$ ) that only diffuse with  $D_f$  and  $D_S \ll D_f$  for which the ACF is the sum of two components of the form of Eq. 3 with correlation times

$$\tau_i = \frac{w_r^2}{4D_i},$$

where  $i = f, S$  (see Materials and Methods). We then use Eqs. 16 and 17 to compute  $\text{var}(\Xi)/\langle \Xi \rangle^2$  for  $\Xi = \overline{N}_i^t, s_i^2$ ,  $i = f, S$  and, by setting  $\text{var}(\Xi)/\langle \Xi \rangle^2 = \alpha^2$ , we derive the convergence times,  $T_\alpha(\Xi)$  for  $\Xi$  to be within its expected value with relative error  $\alpha$ .

Assuming that  $N_f^t$  and  $N_S^t$  obey Poisson statistics and that  $\tau_i \gg \Delta t$ , following this approach we obtain

$$T_\alpha(\overline{N}_i^t) \approx 200\tau_i / \langle N_i^t \rangle \quad \text{and} \quad T_\alpha(s_i^2) \approx 400\tau_i$$

for  $\alpha = 0.1$ . We then see that

$$T_\alpha(\overline{N}_i^t) < T_\alpha(s_f^2)$$

for  $\langle N_i^t \rangle > 1$  and that while  $T_\alpha(\overline{N}_i^t)$  decreases with  $\langle N_i^t \rangle$ , the value  $T_\alpha(s_f^2)$  is independent of this number. Thus, there is a limit to the accuracy with which the fluctuation variances and, thus, the weight of the ACF can be computed after a time  $T_{\text{obs}}$ , which is determined by the correlation times,  $\tau_f$  and  $\tau_S$ , and is independent of the concentrations. Furthermore, this limit dominates the relative error of  $\tilde{G}_o$  if  $\langle N^t \rangle$  is large enough ( $\geq 5$ ). In view of Eq. 19 and considering the slowest correlation time of the example,  $\tau_S$ , we conclude that we must have  $T_{\text{obs}} > \tau_S/\alpha^2 \gg \tau_S$  to estimate  $G_o$  with relative error  $\alpha$ . Although  $\tau_S \gg \tau_f$ , the relative ordering between  $T_\alpha(s_f^2)$  and  $\tau_S$  is arbitrary. The value  $\tau_S$  also determines the time over which  $N_S^t$  changes significantly. Thus, if  $\tau_S \gg T_\alpha(s_f^2)$ , we expect that there would be a range of  $T_{\text{obs}}$  values for which the ACF can be approximated by a single component with correlation time,  $\tau_f$  and weight

$$\tilde{G}_o \sim s_f^2 / \left( \overline{N}_f^t + N_S^t(t=0) \right).$$

Otherwise, the lack of convergence of the ACF computed with  $T_{\text{obs}} < \tau_S$  would be noticeable (e.g., by becoming negative for certain lag times,  $\tau$ ).

This behavior is confirmed by the stochastic numerical simulations of Fig. 2 a, where we plot the ACFs obtained using  $T_{\text{obs}}$  values that satisfy  $T_\alpha(s_f^2) = 0.01 \text{ s} < T_{\text{obs}} = 0.027 \text{ s} \ll \tau_S = 2.65 \text{ s}$  (squares),  $T_{\text{obs}} = 0.87 \text{ s} \sim \tau_S/3$  (triangles), and  $T_{\text{obs}} = 350 \text{ s} \sim 130 \tau_S$  (circles) for  $\alpha = 0.1$ . The ACFs obtained for  $T_{\text{obs}}$  up to  $\sim 0.1\tau_S$  are similar to the one displayed with circles. As may be observed, the correlation time,  $\tau_S$ , is unobservable for  $T_{\text{obs}} \ll \tau_S$  and becomes apparent for  $T_{\text{obs}} \lesssim \tau_S$ . The total weight depends on  $N_{ST}(t=0)$  for  $T_{\text{obs}} \ll \tau_S$  and converges to its actual value,

from which a reliable estimate of the total concentration of fluorescent particles can be derived, for  $T_{\text{obs}} \gg \tau_S$ .

#### Reaction-diffusion system with immobile binding sites

For  $D_S = 0$ , the timescale,  $\tau_S$ , is absent from the ACF and the variance of  $N^t$  is reduced both for the system of Fig. 2 a and for the reaction-diffusion system with respect to the  $D_S \neq 0$  case. These two features imply that  $\Delta\tilde{G}_o/\tilde{G}_o$  is reduced and that the expected value of  $\tilde{G}_o$  may be achieved on a shorter timescale if  $D_S = 0$ . This is equivalent to the transient situation of Fig. 2 a for  $T_{\text{obs}} \ll \tau_S$ , but with  $\tilde{G}_o$  converging to its actual value. This is illustrated in Fig. 2 b, where we show the ACF derived from the same data as in Fig. 1 a ( $D_S = 0$ ) but for values of  $T_{\text{obs}}$  that, when compared with the slowest correlation time of the ACF (in this case,  $\tau_{sm}$ ), satisfy  $T_{\text{obs}}/\tau_{sm} \sim 3$  (crosses), 43 (squares), 681 (circles), and 5450 (triangles). The relative errors of  $\tilde{G}_o$  in Fig. 2 b agree fairly well with the estimates obtained using Eq. 19 with  $\text{var}(N_i^t)$  and  $\text{var}(s_i^2)$  given, respectively, by Eqs. 16 and 17 with  $N^t = N_i^t$  ( $i = f, S$ ) and  $\tau_{\text{corr}} = \tau_{sm}$ . Namely, for the parameters of the simulation, it is

$$\text{var}(N_b^t) = \langle N_b^t \rangle (1 - [P_b^t]/[S]_T) = 10,400$$

and

$$\text{var}(N_f^t) = \langle N_f^t \rangle = 3580,$$

which yield

$$\begin{aligned} \Delta G_o/G_o &\sim 8\tau_{sm}/T_{\text{obs}} \frac{\left(\text{var}(N_f^t)\right)^2 + \left(\text{var}(N_b^t)\right)^2}{\left(\text{var}(N_f^t) + \text{var}(N_b^t)\right)^2} \\ &\sim 5\tau_{sm}/T_{\text{obs}}. \end{aligned}$$

Thus, according to this estimate, the relative error of the examples of Fig. 2 b varies between 1.7 and 0.001.

#### Reaction-diffusion system with slowly moving binding sites

For the reaction-diffusion system with  $D_S \neq 0$ , the slowest correlation time,  $\tau_S$ , also rules the variation of  $N_{ST}$  and determines for how long there is an apparent correlation that makes  $\tilde{G}_o \approx 0$ . Differently from the case with two freely diffusing and equally fluorescent species described before, in this case there is not a one-to-one correspondence between independent random variables and correlation times (see Eq. 7 and Eqs. 11 and 12). But in any case, to compute the error of the total weight estimate,  $\tilde{G}_o$ , we use Eq. 19 with

$$N_1^t \equiv f_i \left( N_f^t + \frac{[S]}{[S]_T} N_b^t \right),$$

$$N_2^t \equiv (1 - f_i) N^t,$$

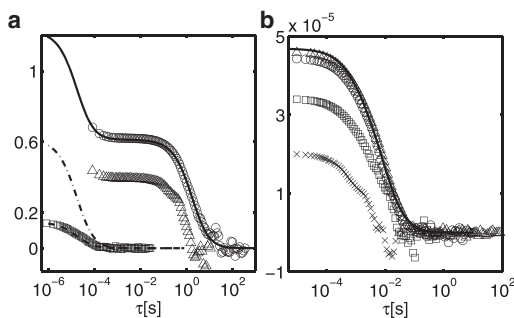


FIGURE 2 ACF,  $G(\tau)$ , computed from stochastic simulations with different  $T_{\text{obs}}$  (symbols) and theoretical function (solid line). (a) System of freely diffusing  $f$  and  $S$  particles with  $D_f = 500 \mu\text{m}^2/\text{s}$ ,  $D_S = 0.005 \mu\text{m}^2/\text{s}$ , and  $\langle N_f \rangle = \langle N_S \rangle = 0.4$ ;  $T_{\text{obs}} = 0.027 \text{ s}$  (squares),  $0.87 \text{ s}$  (triangles), and  $350 \text{ s}$  (circles). Theoretical ACF for  $N_S = 0$  (dashed-dotted line) and rescaled version to match the simulated weight (dashed line). (b) Same system as in Fig. 1 a ( $D_S = 0$ ) for  $T_{\text{obs}} = 0.04 \text{ s}$  (crosses),  $0.64 \text{ s}$  (squares),  $10.22 \text{ s}$  (circles), and  $81.76 \text{ s}$  (triangles).

and

$$N_3^t \equiv \frac{[P_b^t]}{[S]_T} N_b^t.$$

I.e., we work as if  $N_i^t$ ,  $i = 1, 2, 3$ , were three independent random variables, each of which is characterized by a single correlation time ( $\tau_1 = \tau_{\text{coll}}$ ,  $\tau_2 = \tau_{sm}$ , and  $\tau_3 = \tau_S$ ) and such that  $\text{var}(N_i^t) = \langle N_i^t \rangle$ . These variables are such that

$$\sum_i N_i^t = N^t,$$

so that the total weight is given by

$$G_o = \sum_i \text{var}(N_i^t) / \left\langle \left( \sum_i N_i^t \right)^2 \right\rangle$$

as required by Eq. 19. This approximate calculation gives good error estimates as shown in Fig. 3, where we plot the component and total weights obtained from stochastic simulations as functions of  $T_{\text{obs}}$  (symbols), the theoretical values given by Eqs. 11 and 12 (solid lines) and the error of the weights computed as just explained (shaded area). This implies that, as in the case of Fig. 2 a, when particles diffuse and react there is also a basic limit to the convergence time of the weights which is determined by the correlation times (i.e., by the diffusion coefficients and the volume size) and is independent of the

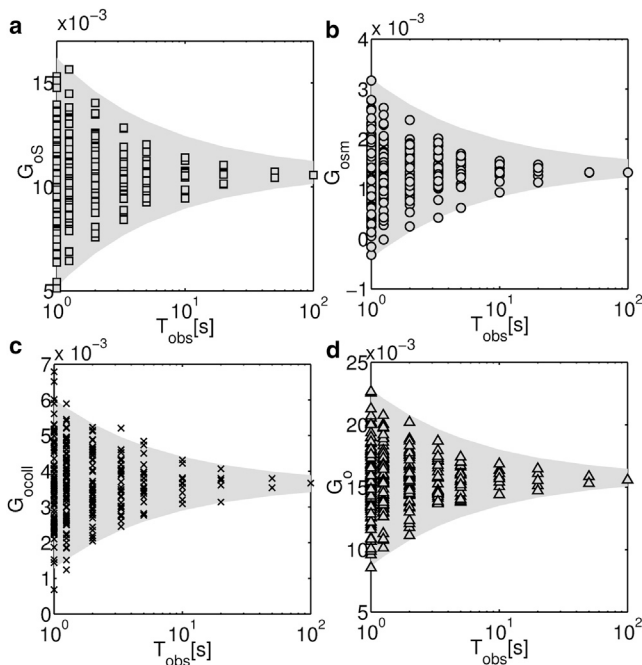


FIGURE 3 Component and total weights of the ACF computed from stochastic simulations of particles that diffuse and react (symbols), corresponding theoretical values (curves), and expected errors around them after an observation time,  $T_{\text{obs}}$ , computed as explained in the text (shaded areas). For simulation parameters, please see Table 1.

mean number of particles in  $V_{\text{obs}}$ . This is a consequence of having

$$\Delta \text{var}(N_i^t) / \langle \text{var}(N_i^t) \rangle^2 = \text{var}(s_i^2) / \langle s_i^2 \rangle^2 \approx 2\rho_i \sim 4\tau_i / T_{\text{obs}},$$

which differs from

$$\text{var}(\bar{N}_i^t) / \langle N_i^t \rangle^2 \approx \rho_i / \langle N_i^t \rangle \sim 2\tau_i / (T_{\text{obs}} \langle N_i^t \rangle)$$

in that it does not depend on the mean number of particles in the observation volume.

## DISCUSSION AND CONCLUSIONS

We have studied the accuracy of concentration estimates that can be derived from the analysis of the ACF obtained from FCS experiments performed on systems of fluorescent particles, those that diffuse and react with mobile or immobile binding sites. We have only considered the limits that fluctuations in the number of observable molecules impose on the observation time needed to obtain such estimates with a given relative error. In actual experiments, the total weight of the ACF,  $G_o$ , is also affected by the noise of the photon-counting process (shot noise). This noise is associated to fluctuations in the number of detected photons per fluorescent molecule and is independent of the underlying dynamics of the observed particles. The effect of this noise has been studied in Krichevsky and Bonnet (3), Koppel (21), and Qian (22), where it has been shown that it affects the ACF at  $\tau = 0$ . Thus, the weights from which the concentrations can be computed are directly affected by this noise. Our calculations should then be considered as providing a lower bound on the estimated concentration error. In most experimental situations, however, the shot noise can be reduced considerably by a proper choice of the experimental parameters.

We have obtained analytic expressions for the weights and their relative errors for the reaction-diffusion system in the fast reaction limit (i.e., when the reaction and diffusion timescales satisfy  $\tau_r \ll \tau_D$  (14,16)). We have verified the analytic results by means of stochastic numerical simulations. In particular, we have shown that when the fluorescent molecules react with immobile ( $D_S = 0$ ) binding sites, the site-bound molecules follow a multinomial instead of a Poisson distribution. This reduces the (observed) fluorescence variance by a finite amount with respect to the case with moving binding sites, as illustrated in Fig. 1 a. Moreover, the variance and the mean of the number of fluorescent molecules in  $V_{\text{obs}}$  ( $\langle N^t \rangle$ ) are no longer equal and the total weight of the ACF,  $G_o$ , is not the inverse of  $\langle N^t \rangle$ . Thus, a blind fit of the data assuming Poisson statistics would result in an erroneous concentration estimate in this case.

We have  $G_o \neq 1/\langle N^t \rangle$  for a system with equally fluorescent noninteracting species if one of them is immobile, as illustrated in Fig. 2 a. This implies that  $G_o \neq 1/\langle N^t \rangle$  for



the reaction-diffusion system with  $D_S = 0$  in the fast diffusion limit (when  $\tau_D \ll \tau_r$ ) because its ACF coincides with that of the noninteracting particle system (14). Given that the same result is obtained in the two limiting situations of the reaction-diffusion system we expect it to hold in any other situation ( $\tau_r \sim \tau_D$ ) as well.

The  $D_S = 0$  case corresponds to a singular limit. Namely, the ACF of the reaction-diffusion system with  $D_S = 0$  in the fast reaction limit is characterized by two correlation times,  $\tau_{\text{coll}}$  and  $\tau_{sm}$ , and  $G_o \neq 1/\langle N^i \rangle$ . As soon as  $D_S \neq 0$ , the ACF has three correlation times,  $\tau_{\text{coll}}$ ,  $\tau_{sm}$ , and  $\tau_S$ , and the Poisson statistics and the inverse relationship between  $G_o$  and  $\langle N^i \rangle$  are recovered. However, from the observation of the fluctuations, only estimates of the variance and mean of the number of fluorescent molecules can be derived. To have accurate estimates, the system must be observed for a long enough time. To determine how long is sufficient, we first explored this problem for the simpler system of two freely diffusing equally fluorescent species, as illustrated in Fig. 2 a.

We obtained that the times,  $T_\alpha(\overline{N}_i^f)$  and  $T_\alpha(s_i^2)$ , needed to estimate the mean and the variance of the number of fluorescent molecules of each species,  $N_i^f$ , with relative error  $\alpha = 0.1$ , were approximately given by

$$T_\alpha(\overline{N}_i^f) \approx 200\tau_i / \langle N_i^f \rangle \text{ and } T_\alpha(s_i^2) \approx 400\tau_i$$

with  $\tau_i$  as the diffusive correlation time of each species. This implies that the system must be observed for hundreds of correlation times,  $\tau_i$ , to derive reliable concentration estimates from the ACF (with  $\sim 10\%$  error). The fact that  $T_\alpha(s_i^2)$  is independent of  $\langle N_i^f \rangle$ , on the other hand, sets a limit for the relative error of the variance estimate,  $s_i^2$ , and, thus, the weight of the ACF, which is independent of the concentrations.

This concentration-independent limit dominates the relative error of  $\hat{G}_o$  if  $\langle N^i \rangle$  is moderately large ( $\geq 5$ ). In such a case, changing the number of fluorescent particles in the observation volume would not result in an improvement of the accuracy of the weight estimate. As mentioned before, these results hold for the reaction-diffusion system in the fast diffusion limit as well. The illustration of Fig. 3, on the other hand, shows that similar expressions for the weight-relative errors as those of the system with noninteracting species can be used in the case of the reaction-diffusion system with  $D_S \neq 0$  in the fast reaction limit. This also implies that, in this limit, the time needed for the weight to be within its actual value with  $\alpha = 0.1$  relative error is  $\sim 400$  times that of the slowest correlation time,  $\tau_S$ , in this case. As before, given that we obtain similar results for the reaction-diffusion system in its two limiting situations, we expect that the same results also hold for any other intermediate situation.

Because the concentration estimates are derived from the weights, this means that a much longer observation time is

necessary to derive them than to estimate correlation times. However, depending on  $D_S$ , observing the system for such a long time could be unattainable in an actual experiment, due to bleaching or other causes. For example, the duration of FCS experiments performed in *Drosophila melanogaster* embryos to estimate the diffusion coefficient of Bcd is limited by the time between successive nuclear divisions ( $\sim 8$  min). In Abu-Arish et al. (20) the ACF derived from such FCS experiments was computed for lag times,  $\tau$ , between  $10 \mu\text{s}$  and  $15$  s. The slowest correlation time derived from the experiment was  $\sim 422$  ms  $\pm$  164 ms (our  $\tau_S$ ). The ratio  $\tau_S/T_{\text{obs}}$  for  $T_{\text{obs}} = 15$  s is  $\sim 0.03$ , in which case, according to our calculation, the relative error of the weight,  $G_{oS}$ , is  $>33\%$ .

A larger relative error can then be expected for concentration estimates derived from the total weight of the ACF. It must be noted that in this example the particles (Bcd) are not uniformly distributed in the embryo. Their distribution within  $V_{\text{obs}}$ , however, is approximately uniform ( $w_r \sim 0.3 \mu\text{m}$ , while the characteristic length-scale of the Bcd gradient is  $\sim 100 \mu\text{m}$ ), so our theory can be applied (17).

The fact that the expressions for the errors derived for the case with noninteracting species also provide good estimates for the reaction-diffusion system in the fast reaction limit, allows us to extrapolate to the latter some of the results of Fig. 2 a that were obtained for the system with two freely diffusing species. This is easier to do if we assume that all particles,  $P$ , of the reaction-diffusion system are fluorescent ( $f_i = 1$ ). In such a case the ACF, in the fast reaction limit, has only two timescales,  $\tau_S$  and  $\tau_{\text{coll}}$  (see Eq. 11), as in the example with two freely diffusing species and  $D_S \neq 0$ . In such a case, if  $\tau_S$  and  $\tau_{\text{coll}}$  are sufficiently different so that  $\tau_S \gg T_\alpha(s_{\text{coll}}^2) \gg \tau_{\text{coll}}$  for  $\alpha = 1$ , experiments with  $T_{\text{obs}} \sim T_\alpha(s_{\text{coll}}^2)$  would provide an approximated ACF in which the timescale,  $\tau_S$ , would go undetected as in one of the examples of Fig. 2 a. If the separation is not wide enough, however, the presence of  $\tau_S$  would be apparent as in another of the examples of Fig. 2 a.

The above discussion shows that accurate concentration estimates require a much longer observation time than the (diffusive) correlation times when the sites diffuse very slowly. This long observation time cannot be reduced by changing  $V_{\text{obs}}$  (unless  $V_{\text{obs}}$  becomes comparable to the accessible volume,  $V_T$ ). If exactly the same system but with immobile binding sites is probed, a good estimate can be achieved in a shorter time, as illustrated in Fig. 2 b, because the long-time correlation introduced by the slowly diffusing sites disappears. The value that is derived in this case, however, depends on the total number of binding sites that are inside  $V_{\text{obs}}$ . If this number is very different from  $[S]_T V_{\text{obs}}$  with  $[S]_T$  as the equilibrium concentration over the accessible volume, then the experiment is exploring some sort of local equilibrium and provides information about it.

In the simulations of reaction-diffusion systems discussed in this article, the relative error of the total weight of the

ACF after a time,  $T_{\text{obs}}$ , is dominated by that of the variance. The same correlation times that enter this relative error are also involved in that of the concentrations. The accuracy with which concentrations are sensed is relevant for the processing of information in cells. For this problem, the distinction between fluorescent and nonfluorescent particles is unnecessary. In this case, the two correlation times involved in the accuracy of the concentration estimate are the one of the binding sites if the sites are mobile,  $\tau_s$ , and the one associated to the free,  $\tau_f$ , or the collective diffusion coefficient,  $\tau_{\text{coll}}$  in the fast diffusion and the fast reaction limits, respectively (14). If the sites are immobile, only one correlation time remains:  $\tau_{\text{coll}}$  (which can be much shorter than  $\tau_{sm}$  (15)), or  $\tau_f$ . This implies that the diffusion time of the moving particles is the one that sets a lower bound for the observation time required by endogenous sensors to detect effector concentrations with a given accuracy. This result agrees with those obtained in Bialek and Setayeshgar (11,13) and Gregor et al. (12). We finally remark that the limited volume over which some molecules move inside cells can also reduce the variance of their number, as illustrated in Fig. 1 b.

In this example, the change of  $V_{\text{obs}}/V_T$  between both curves is such that only an ~4% variation of the total weight is expected. In fact, the simulations give  $G_o = 5.94 \times 10^{-5}$  for  $V_T = 8 \mu\text{m}^3$ , and  $G_o = 6.18 \times 10^{-5}$  for  $V_T = 64 \mu\text{m}^3$ , which agree with the theory. For other parameter values a larger reduction could be achieved. Such restriction could then shorten the time required for their concentration to be read accurately by the endogenous sensing mechanisms.

## SUPPORTING MATERIAL

Additional supplemental information including 14 equations is available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(14\)01132-1](http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)01132-1).

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Supporting information for “For how long should a system be observed to obtain reliable concentration estimates from the measurement of fluctuations”

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# 1 Supporting information

## 1.1 Computation of the autocorrelation function (ACF)

We consider a system composed of fluorescently tagged and untagged molecules of the same species,  $P_f^t$  and  $P_f^u$ , that diffuse with free diffusion coefficient  $D_f$  and interact with binding sites,  $S$ , forming the bound species,  $P_b^t$  and  $P_b^u$ , respectively. An appropriate assumption is that the binding sites belong to very massive molecules so that  $S$ ,  $P_b^t$  and  $P_b^u$  diffuse with the same coefficient,  $D_s \ll D_f$ . The reaction scheme is given by:



where  $k_{on}$  and  $k_{off}$  are the rates of binding and unbinding respectively and define the dissociation constant,  $K_D = \frac{k_{off}}{k_{on}}$ .

The fluorescence collected by the microscope at time  $t$  is  $F(t) = \Delta t \int d^3\vec{r} I(\vec{r}) q(c_f^t(\vec{r}, t) + c_b^t(\vec{r}, t))$ ,  $\Delta t$  is the sampling time,  $I(\vec{r})$ , is the distribution of the excitation light in the sample,  $q$  is the product of the absorption cross section by the fluorescence quantum yield and the efficiency of the fluorophore, and  $c_f^t$ ,  $c_b^t$  are the number densities of fluorescent molecules in the free and bound forms. We assume that the fluorescent molecules have the same quantum yield in their free and bound forms. The autocorrelation function (ACF) is defined and computed as:

$$G(\tau) \equiv \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \approx \Delta t \frac{\int \delta F(t) \delta F(t + \tau) dt}{(\int F(t) dt)^2}, \quad (2)$$

where  $\delta F(t) = F(t) - \langle F \rangle$ , is the deviation of the fluorescence from its mean,  $\langle F \rangle$ , which is approximated by the average as shown above. An analytic expression in terms of integrals can be obtained for  $G(\tau)$  in the case of a system as the one considered here. To this end we follow the procedure described in (1). We start with the approximation to the master equation

of the system that is given by the reaction-diffusion equations:

$$\begin{aligned}
\frac{\partial c_f^t}{\partial t} &= D_f \nabla^2 c_f^t - k_{on} c_f^t c_S + k_{off} c_b^t, \\
\frac{\partial c_b^t}{\partial t} &= D_S \nabla^2 c_b^t + k_{on} c_f^t c_S - k_{off} c_b^t, \\
\frac{\partial c_f^u}{\partial t} &= D_f \nabla^2 c_f^u - k_{on} c_f^u c_S + k_{off} c_b^u, \\
\frac{\partial c_b^u}{\partial t} &= D_S \nabla^2 c_b^u + k_{on} c_f^u c_S - k_{off} c_b^u, \\
\frac{\partial c_S}{\partial t} &= D_S \nabla^2 c_S - k_{on} c_f c_S + k_{off} c_b,
\end{aligned} \tag{3}$$

where  $c_S$  is the number density of free binding sites, and  $c_f = c_f^t + c_f^u$  and  $c_b = c_b^t + c_b^u$  are the total concentrations of free and bound particles, respectively and we have used mass action kinetics to describe the reactions. We then linearize Eqs. (3) around the equilibrium solution,  $c_{f,eq}^{u,t} \equiv [P_f^{u,t}]$ ,  $c_{b,eq}^{u,t} \equiv [P_b^{u,t}]$ ,  $c_{S,eq} \equiv [S]$ , that satisfies  $[P_f^t][S] = K_D[P_b^t]$ ,  $[P_f^u][S] = K_D[P_b^u]$ , and write the ACF as:

$$G(\tau) = \frac{\int d^3 \vec{r} d^3 \vec{r}' I(\vec{r}) I(\vec{r}') \sum_{i,j} q_i q_j \langle \delta c_i(\vec{r}, 0) \delta c_j(\vec{r}', \tau) \rangle}{\left( q V_{obs} ([P_f^t] + [P_b^t]) \right)^2}, \tag{4}$$

where we have used the notation,  $\delta c_i$ , for the deviation of the concentration with respect to equilibrium of the  $i$ -th species and  $q_i$  for the corresponding fluorescence efficiency (which is either zero or  $q$ ) and where  $V_{obs} = \frac{\int d^3 \vec{r} I(\vec{r})}{I(0)}$ . In order to find the deviations as functions of space and time we Fourier transform the linearized reaction-diffusion equations. The transformed system can be expressed as  $\dot{C}_\xi = M_\xi C_\xi$ , with  $C_\xi$  the column vector of deviations with respect to equilibrium in Fourier space (the transpose of  $((\delta c_f^t)_\xi, (\delta c_b^t)_\xi, (\delta c_S)_\xi, (\delta c_f^u)_\xi, (\delta c_b^u)_\xi)$ ),  $\vec{\xi}$  the wavenumber (the variable conjugate to the position,  $\mathbf{r}$ , in real space) and  $M_\xi$  given by:

$$M_\xi = \begin{bmatrix} -D_f \xi^2 - k_{on}[S] & k_{off} & -k_{on}[P_f^t] & 0 & 0 \\ k_{on}[S] & -D_s \xi^2 - k_{off} & k_{on}[P_f^t] & 0 & 0 \\ -k_{on}[S] & k_{off} & -D_s \xi^2 - k_{on}[P_f^t] - k_{on}[P_f^u] & -k_{on}[S] & k_{off} \\ 0 & 0 & -k_{on}[P_f^u] & -D_f \xi^2 - k_{on}[S] & k_{off} \\ 0 & 0 & k_{on}[P_f^u] & k_{on}[S] & -D_s \xi^2 - k_{off} \end{bmatrix}, \tag{5}$$

We then seek a solution of the linear system in Fourier space. To this end

we look for eigenvalues and eigenvectors of  $M_\xi$ . The eigenvalues are:

$$\begin{aligned}
\lambda_1 &= -D_s \xi^2, \\
\lambda_2 &= -\frac{1}{2} \left( k_{off} + k_{on}([S] + [P_f]_T) + (D_f + D_s) \xi^2 + \sqrt{\beta_1} \right), \\
\lambda_3 &= -\frac{1}{2} \left( k_{off} + k_{on}([S] + [P_f]_T) + (D_f + D_s) \xi^2 - \sqrt{\beta_1} \right), \\
\lambda_4 &= -\frac{1}{2} \left( k_{off} + k_{on}[S] + (D_f + D_s) \xi^2 + \sqrt{\beta_2} \right), \\
\lambda_5 &= -\frac{1}{2} \left( k_{off} + k_{on}[S] + (D_f + D_s) \xi^2 - \sqrt{\beta_2} \right),
\end{aligned} \tag{6}$$

where  $\beta_1 = (k_{off} + k_{on}([S] + [P_f]_T))^2 + 2(D_f - D_s)\xi^2(k_{on}[S] - k_{on}[P_f]_T - k_{off}) + (D_f - D_s)^2\xi^4$ ,  $\beta_2 = (k_{off} + k_{on}[S])^2 + 2(D_f - D_s)\xi^2(k_{on}[S] - k_{off}) + (D_f - D_s)^2\xi^4$  and  $[P_f]_T = [P_f^t] + [P_f^u]$ . The matrix of eigenvectors is:

$$X = \begin{bmatrix} 0 & \frac{[P_f^t]}{[P_f^u]} \frac{\phi_2}{k_{on}[S]} & \frac{[P_f^t]}{[P_f^u]} \frac{\phi_3}{k_{on}[S]} & -\frac{\phi_4}{k_{on}[S]} & -\frac{\phi_5}{k_{on}[S]} \\ \frac{[P_f^t]}{[P_f^u]} & \frac{[P_f^t]}{[P_f^u]} & \frac{[P_f^t]}{[P_f^u]} & -1 & -1 \\ K_D & -\frac{[P_f]_T}{[P_f^u]} & -\frac{[P_f]_T}{[P_f^u]} & 0 & 0 \\ 0 & \frac{\phi_2}{k_{on}[S]} & \frac{\phi_3}{k_{on}[S]} & \frac{\phi_4}{k_{on}[S]} & \frac{\phi_5}{k_{on}[S]} \\ 1 & 1 & 1 & 1 & 1 \end{bmatrix} \tag{7}$$

where  $\phi_2 = \lambda_2 + k_{off} + k_{on}[P_f]_T$ ,  $\phi_3 = \lambda_3 + k_{off} + k_{on}[P_f]_T$ ,  $\phi_3 - \phi_2 = \sqrt{\beta_1}$ ,  $\phi_4 = \lambda_4 + k_{off}$ ,  $\phi_5 = \lambda_5 + k_{off}$  and  $\phi_5 - \phi_4 = \sqrt{\beta_2}$ . Each component,  $i$ , of the vector,  $C_\xi(t)$ , can be written as  $(C_\xi(t))_i = \sum_{j,\ell} X_{ij}(X^{-1})_{j\ell}(C_\xi(0))_\ell \exp(\lambda_j t)$ . Replacing this expression in Eq. (4) we observe that the ACF depends on the initial correlations between the particle number fluctuations,  $\delta N_i = N_i - \langle N_i \rangle$ , of the various species ( $1 \leq i \leq 5$ ). Here we assume that the correlation distance is short ranged so that  $\langle \delta c_i(\vec{r}, 0) \delta c_j(\vec{r}', 0) \rangle \propto \delta(\vec{r} - \vec{r}')$ . In (1) it is further assumed that  $\langle \delta c_i(\vec{r}, 0) \delta c_j(\vec{r}', 0) \rangle = \langle c_i \rangle \delta_{ij} \delta(\vec{r} - \vec{r}')$ , *i.e.*, that fluctuations in the particle numbers of different species are initially uncorrelated and that each of them obeys Poisson statistics. Here we assume instead that  $\langle \delta c_i(\vec{r}, 0) \delta c_j(\vec{r}', 0) \rangle = \sigma_{ij}^2 \delta(\vec{r} - \vec{r}') / V_{obs}$  with a particle number correlation matrix,  $\sigma_{ij}^2 \equiv \langle \delta N_i \delta N_j \rangle$  that is not necessarily diagonal. The ACF can then be written as:

$$\begin{aligned}
G(\tau) &= \frac{(2\pi)^{-3}}{q^2 V_{obs} ([P_f^t] + [P_b^t])^2} \\
&\times \int d^3 \xi \hat{I}(\vec{\xi}) \sum_{j,l} q_j q_l \sum_s X_{l,s} \exp(\lambda_s \tau) \sum_k (X^{-1})_{s,k} \sigma_{j,k}^2
\end{aligned} \tag{8}$$

where the subscripts  $j, l$ , label the 5 species of the reaction-diffusion system, the index,  $s$ , labels the eigenvalues,  $X^{-1}$  is the inverse of the eigenvectors matrix and  $\hat{I}(\vec{\xi})$  is the Fourier transform of  $I(\vec{r})$ . Given that  $\sigma^2$  is a symmetric matrix, we can define  $Y = X^{-1}\sigma^2$ . Taking into account that only particles in their tagged form are fluorescent and that both the free and bound ones have the same  $q$ , Eq. 8 can be reduced to:

$$G(\tau) = \frac{(2\pi)^{-3}}{q^2 V_{obs} ([P_f^t] + [P_b^t])^2} \int d^3\xi I(\vec{\xi}) \sum_i \alpha_i e^{\lambda_i \tau}, \quad (9)$$

with

$$\begin{aligned} \alpha_1 &= q^2 (X_{11} + X_{21})(Y_{11} + Y_{12}), \\ \alpha_2 &= q^2 (X_{12} + X_{22})(Y_{21} + Y_{22}), \\ \alpha_3 &= q^2 (X_{13} + X_{23})(Y_{31} + Y_{32}), \\ \alpha_4 &= q^2 (X_{14} + X_{24})(Y_{41} + Y_{42}), \\ \alpha_5 &= q^2 (X_{15} + X_{25})(Y_{51} + Y_{52}). \end{aligned} \quad (10)$$

Notice from Eq. 9, that the ACF can be expressed as the sum of several components, one for each eigenvalue. Nevertheless, it is not always possible to obtain an analytical expression for it. In (2), (3), it is shown that there exist some limits for which the ACF can be expressed as the sum of diffusive components. One of them is the so called *fast reaction* limit, in which the reactions occur on a much faster time scale than the time it takes for molecules to cross the observation volume. In this limit, the ACF reduces to the sum of three diffusive components which can be expressed as:

$$\begin{aligned} G(\tau) &= \frac{G_{oS}}{\left(1 + \frac{\tau}{\tau_S}\right) \sqrt{1 + w^2 \frac{\tau}{\tau_S}}} + \frac{G_{oColl}}{\left(1 + \frac{\tau}{\tau_{Coll}}\right) \sqrt{1 + w^2 \frac{\tau}{\tau_{Coll}}}} \\ &\quad + \frac{G_{oS_m}}{\left(1 + \frac{\tau}{\tau_{S_m}}\right) \sqrt{1 + w^2 \frac{\tau}{\tau_{S_m}}}}, \end{aligned} \quad (11)$$

where we have considered a gaussian illumination profile,  $I(\vec{r}) = I_0 \exp\left(-\frac{2(x^2+y^2)}{w_r^2} - \frac{2z^2}{w_z^2}\right)$ , with  $w = w_z/w_r$  the ratio of the beam waist along the direction of propagation of light,  $w_z$ , and the perpendicular direction  $w_r$ . The weights and

characteristic times of each component are:

$$\begin{aligned}
G_{os} &= \frac{1}{\langle N^t \rangle^2} \frac{[P_f^t]}{K_D + [P_f]_T} \gamma_1, \\
G_{ocoll} &= \frac{1}{\langle N^t \rangle^2} \frac{[P_f^t]}{K_D + [P_f]_T} \frac{K_D \gamma_2 - [P_f]_T \gamma_3}{[P_f]_T}, \\
G_{osm} &= \frac{1}{\langle N^t \rangle^2} \frac{[P_f^u] \gamma_4 - [P_f^t] \gamma_5}{[P_f]_T}, \quad \tau_S = \frac{w_r^2}{4D_S}, \\
\tau_{coll} &= \frac{w_r^2 (1 + \frac{[S]^2}{K_D [S]_T})}{4(D_f + \frac{[S]^2}{K_D [S]_T} D_S)}, \quad \tau_{sm} = \frac{w_r^2 (1 + \frac{[S]}{K_D})}{4(D_f + \frac{[S]}{K_D} D_S)}, \quad (12)
\end{aligned}$$

where  $\langle N^t \rangle = ([P_f^t] + [P_b^t])V_{obs}$  is the mean total number of fluorescent particles in the observation volume,  $[S]_T$  is the total concentration of binding sites and the various  $\gamma_i$  are correlation factors between the species derived from Eq. 8,

$$\begin{aligned}
\gamma_1 &= \langle \delta N_b^{t2} \rangle + \langle \delta N_f^t \delta N_b^t \rangle + \langle \delta N_f^t \delta N_S \rangle \\
&+ \langle \delta N_f^t \delta N_b^u \rangle + \langle \delta N_b^t \delta N_b^u \rangle + \langle \delta N_b^t \delta N_S \rangle, \\
\gamma_2 &= \langle \delta N_f^{t2} \rangle + \langle \delta N_b^{t2} \rangle + 2\langle \delta N_f^t \delta N_b^t \rangle + \\
&+ \langle \delta N_f^t \delta N_f^u \rangle + \langle \delta N_f^u \delta N_b^t \rangle + \langle \delta N_f^t \delta N_b^u \rangle + \langle \delta N_b^t \delta N_b^u \rangle, \\
\gamma_3 &= \langle \delta N_f^t \delta N_S \rangle + \langle \delta N_b^t \delta N_S \rangle - \langle \delta N_f^{t2} \rangle \\
&- \langle \delta N_f^t \delta N_b^t \rangle - \langle \delta N_f^t \delta N_f^u \rangle - \langle \delta N_f^u \delta N_b^t \rangle, \\
\gamma_4 &= \langle \delta N_f^{t2} \rangle + \langle \delta N_b^{t2} \rangle + 2\langle \delta N_f^t \delta N_b^t \rangle, \\
\gamma_5 &= \langle \delta N_f^t \delta N_f^u \rangle + \langle \delta N_f^u \delta N_b^t \rangle + \langle \delta N_f^t \delta N_b^u \rangle \\
&+ \langle \delta N_b^t \delta N_b^u \rangle, \quad (13)
\end{aligned}$$

### 1.1.1 Uncorrelated Species

The general expression given by Eqs. (12)–(13) embraces several cases. Let us consider first the usual case in which the species are not correlated and obey Poisson statistics, *i.e.*  $\langle \delta N_i \delta N_j \rangle = \langle N_i \rangle \delta_{ij}$ . The weights of the compo-



nents then become (2):

$$\begin{aligned}
 G_{os} &= \frac{[P_b^t]^2}{V([P_f^t] + [P_b^t])^2[S]_T}, \\
 G_{ocoll} &= \frac{[P_f^t]}{V([P_f^t] + [P_b^t])^2} f_t \left( 1 + \frac{[S]^2}{K_D[S]_T} \right), \\
 G_{osm} &= \frac{1 - f_t}{V([P_f^t] + [P_b^t])},
 \end{aligned} \tag{14}$$

where  $f_t \equiv [P_f^t]/([P_f^t] + [P_b^t]) = [P_b^t]/([P_b^t] + [P_b^u])$  is the fraction of tagged molecules at equilibrium.

### 1.1.2 Immobile binding sites

We now consider the same case as before but with the constraint that the binding sites do not diffuse, *i.e.*  $D_S = 0$ . The number of binding sites inside the observation volume is then fixed,  $N_S + N_b^t + N_b^u = N_{S_T}$ , thus  $\delta N_b^t + \delta N_b^u + \delta N_S = 0$ . Therefore  $N_b^t$ ,  $N_b^u$  and  $N_S$  are correlated, following a multinomial distribution such that  $\langle \delta N_S \delta N_b^t \rangle + \langle \delta N_S \delta N_b^u \rangle = -\langle \delta N_S^2 \rangle$ . There are no other correlations in the system. From Eq. 13 it can be seen that  $\gamma_1 = 0$ , causing the weight associated to  $D_S$  to become exactly zero, *i.e.*,  $G_{oS} = 0$ . The other  $\gamma_i$ s remain the same as in the previous case, so that the other two weights do not change.

### 1.1.3 Confined Volume

If the total volume where the particles are allowed to move,  $V_T$ , is not much larger than the observation volume,  $V_T \gtrsim V_{obs}$ , the number of particles inside  $V_{obs}$  is correlated with the number of particles outside. The distribution of particles of each type inside and outside the volume follows a Binomial distribution with parameter  $p = \frac{V_{obs}}{V_T}$ . Then the variance of the number of particles of each species is  $\langle \delta N_i^2 \rangle = \langle N_i \rangle (1 - \frac{V_{obs}}{V_T})$ . Replacing this expression in Eqs. (12)–(13) we observe that the resulting ACF is the same as in the  $V_T \gg V_{obs}$  but multiplied by  $1 - p$ .

## Supporting References

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