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Distinguishing Positional Uncertainty from True Mobility in Single-Molecule Trajectories That Exhibit Multiple Diffusive Modes

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Abstract

Although imperfect spatial localization in single-molecule object tracking experiments has long been recognized to induce apparent motion in an immobile population of molecules, this effect is often ignored or incorrectly analyzed for mobile molecules. In particular, apparent motion due to positional uncertainty is often incorrectly assigned as a distinct diffusive mode. Here we show that, due to both static and dynamic contributions, positional uncertainty does not introduce a new apparent diffusive mode into trajectories, but instead causes a systematic shift of each measured diffusion coefficient. This shift is relatively simple: a factor of $\sigma^2/\Delta t$ is added to each diffusion coefficient, where σ is the positional uncertainty length scale and Δt is the time interval between observations. Therefore, by calculating the apparent diffusion coefficients as a function of Δt , it is straightforward to separate the true diffusion coefficients from the effective positional uncertainty. As a concrete demonstration, we apply this approach to the diffusion of the protein fibrinogen adsorbed to a hydrophobic surface, a system that exhibits three distinct modes of diffusion.

Keywords

diffusion; total internal reflection fluorescence microscopy (TIRFM); fibrinogen; static error; dynamic error; single molecule

Introduction

One important use of single-molecule tracking techniques is to provide a quantitatively accurate picture of heterogeneous diffusion that often takes the form of a random walk with multiple, interspersed modes. Parameters characterizing diffusion can subsequently be interpreted to understand such systems as cell membrane structure (Fujiwara et al., 2002), intracellular transport (Yildiz et al., 2004), and molecular interactions with solid-liquid (Honciuc et al., 2009; Honciuc & Schwartz, 2009; Kastantin & Schwartz, 2011) or liquid-liquid interfaces (Walder et al., 2010; Walder & Schwartz, 2010).

The inability to localize an object with perfect spatial and temporal precision presents a barrier to the quantitative understanding of heterogeneous diffusion. This error can be divided into two types: static and dynamic (Savin & Doyle, 2005). Static error stems from limits on instrumental precision whereby an immobile object may be assigned different positions when measured at two different times. This error also affects slowly moving objects if their displacement in a given time interval is comparable to the instrumental

precision. In single-molecule tracking, static errors depend both on the signal-to-noise ratio (SNR) and the algorithm used to calculate the subdiffraction object position (Bobroff, 1986; Thompson et al., 2002). Good SNRs and Gaussian fits to two-dimensional (2D) intensity profiles have demonstrated static localization errors below 20 nm (Wu et al., 2008). On the other hand, dynamic errors occur due to the motion of the object itself. For example, Brownian motion is studied at the single-molecule level by measuring the average object position over a fixed time interval, typically an image acquisition time (t_{ac}). This average position is then used to approximate an object's initial position during the acquisition; however, the error in this approximation increases when the molecule diffuses more quickly during a particular image acquisition interval.

To minimize dynamic error, it is best to use short t_{ac} and to observe object positions with a long lag time, Δt , such that $\Delta t \gg t_{ac}$. However, many biological systems require the use of suboptimal fluorophores (e.g., fluorescent proteins) and can have high levels of background fluorescence leading to low SNRs when using short t_{ac} (Sako et al., 2000), thereby increasing static error. High static error can also result from high throughput tracking methods, which rely on fast but less accurate algorithms for object localization (Honciuc & Schwartz, 2009; Walder et al., 2010; Kastantin et al., 2011). Similarly, it is not always feasible to use long Δt to study fast dynamics. For example, resonance energy transfer was used to correlate molecular conformation in single-stranded DNA to diffusive behavior in a time interval of ~ 0.5 s before desorption (Kastantin & Schwartz, 2011). In this work, $t_{ac} = 0.3$ s was required to obtain good signal in multiple channels simultaneously and thus a lag time of $\Delta t \gg t_{ac}$ would have been too long to resolve this phenomenon. Additionally, when studying mechanisms of anomalous diffusion (Bychuk & Oshaughnessy, 1994; Kues et al., 2001), one seeks to observe the variation of apparent diffusion on Δt and must include data acquired using short Δt . Thus, it is exceptional, rather than the typical, to find experimental and analytical conditions in which both static and dynamic errors are negligible.

Researchers have been aware of the effects of positional uncertainty for quite some time, and it is widely recognized that an immobilized population of objects will appear to move with a nonzero diffusion coefficient. In experiments with both mobile and immobile objects, the immobile population is often accounted for by modeling a “fake” mobile population, while no correction is made for the effect of positional uncertainty on “true” mobile populations (Kues et al., 2001). Improper treatment of positional uncertainty can affect conclusions drawn from such analyses. For example, Ciobanasu et al. (2009) concluded that TAT peptides are not completely inserted into the lipid bilayer of anionic giant unilamellar vesicles on the basis of a 30% higher diffusion coefficient of TAT relative to that of liquid-disordered lipids in the bilayer. In this study, TAT diffusion was observed with a threefold shorter Δt than lipid diffusion causing contributions from static error to be unequal in the two analyses. Correction for the effects of positional uncertainty, as described in the current work, would have shown a much smaller difference between the two diffusion coefficients, potentially altering their conclusion. We expect that errors of this type will increase as multimodal analysis of single-molecule tracking data becomes more widely practiced.

This work examines the effect of positional uncertainty on mobile molecules in heterogeneous systems. We present a model to handle positional uncertainty in multimodal diffusion and then apply this result to an experimental system: the protein fibrinogen (Fg), diffusing on hydrophobically-modified fused silica. We demonstrate that, due to both static and dynamic errors, positional uncertainty elevates the apparent diffusion of *all* subpopulations in the system, both mobile and immobile.

Modeling the Effects of Positional Uncertainty

Consider an object in a 2D plane that starts at the origin and moves to a position, (x_o, y_o) , at a later time, Δt . Due to uncertainty in determining each of these positions, one assumes that the probability of observing the object at either an arbitrary initial position, (x_1, y_1) or an arbitrary final position, (x_2, y_2) is given by a 2D Gaussian distribution, centered about its actual position at each time, with standard deviation, σ . As discussed previously, positional uncertainty can be due to both static and dynamic errors. As will become apparent by the end of this section, the assumption of a Gaussian probability distribution for object localization is also implicit in the experimentally-verified scaling of the apparent diffusion coefficient of immobilized molecules with $\sigma^2/\Delta t$ (Kues et al., 2001). For Brownian motion, one also expects interfacial diffusion to follow 2D Gaussian random-walk statistics where the probability (q) of finding the object at position (x_o, y_o) after a lag time of Δt is given by equation (1):

$$q = \sum_{i=1}^N f_i (4\pi D_i \Delta t)^{-1} \exp\left(-\frac{x_o^2 + y_o^2}{4D_i \Delta t}\right). \quad (1)$$

Here, D_i is the diffusion coefficient of mode i and f_i is the relative fraction of steps drawn from one of N modes such that $\sum_{i=1}^N f_i = 1$. Due to uncertainty in measuring each position, the probability of observing an object at initial position, (x_1, y_1) and final position, (x_2, y_2) is given as q_{obs} in equation (2). Note that because the magnitude of the dynamic error depends on the diffusive mode, we have allowed σ to adopt mode-specific values. This is possible because the multimodal squared-displacement distribution is a linear combination of steps drawn from different modes.

$$q_{obs} = \sum_{i=1}^N f_i \frac{\exp\left(-\frac{x_1^2 + y_1^2}{2\sigma_i^2}\right) \exp\left(-\frac{(x_2 - x_1)^2 + (y_2 - y_1)^2}{2\sigma_i^2}\right) \exp\left(-\frac{x_o^2 + y_o^2}{4D_i \Delta t}\right)}{2\pi\sigma_i^2 \cdot 2\pi\sigma_i^2 \cdot 4\pi D_i \Delta t}. \quad (2)$$

To determine q_{obs} as a function of the observed separation, r , between the initial and final position, it is convenient to redefine $x_2 = x_1 - r_x$ and $y_2 = y_1 - r_y$, where $r^2 = r_x^2 + r_y^2$. Next we perform an average over x_1, y_1 space and all potential final positions, x_o and y_o to determine the expected squared-displacement distribution in the presence of positional uncertainty as shown in equation (3):

$$\begin{aligned} \langle q_{obs} \rangle &= (16\pi^3 \Delta t)^{-1} \sum_{i=1}^N \left[(\sigma_i^4 D_i)^{-1} \int \int \int \exp\left(-\frac{x_1^2 + y_1^2}{2\sigma_i^2}\right) \exp\left(-\frac{(x_1 - r_x - x_o)^2 + (y_1 - r_y - y_o)^2}{2\sigma_i^2}\right) \times \exp\left(-\frac{x_o^2 + y_o^2}{4D_i \Delta t}\right) dx_1 dy_1 dx_o dy_o \right] \\ &= \sum_{i=1}^N f_i [4\pi(D\Delta t + \sigma_i^2)]^{-1} \exp\left(-\frac{r^2}{4(D\Delta t + \sigma_i^2)}\right). \end{aligned} \quad (3)$$

Comparing equations (1) and (3) shows that the apparent diffusion coefficient of each mode, $D_{i,app}$, is related to the true diffusion coefficient as shown in equation (4):

$$D_{i,app} = D_i + \sigma_i^2 / \Delta t. \quad (4)$$

Thus, a plot of $D_{i,app}$ versus Δt^{-1} will yield a line with slope σ_i^2 and an intercept that is the true diffusion coefficient.

An important practical consequence of this analysis for modeling and interpreting experimental data is that positional uncertainty serves to increase the apparent diffusion coefficient of *all* diffusive modes in the system by the amount $\sigma_i^2/\Delta t$. Thus, positional uncertainty of several tens of nm and Δt near ~ 0.1 s can significantly elevate diffusion coefficients in the range of $10^{-2} \mu\text{m}^2/\text{s}$ that are commonly observed for macromolecules diffusing on solid surfaces.

Single-Molecule Diffusion of Fibrinogen

We demonstrate the effect of positional uncertainty in an analysis of fluorescently-labeled Fg diffusing on fused silica modified with hydrophobic trimethylsilane (TMS). This system, including surface preparation and data acquisition using total internal reflection fluorescence microscopy, has been described previously (Kastantin et al., 2011), and this reference should be consulted for full experimental details. In the current experiments $t_{ac} = 50$ ms and the temperature was maintained at $25.0 \pm 0.1^\circ\text{C}$. Object positions were calculated as the centroid of intensity, and object tracking was accomplished by identifying the closest objects in sequential frames while requiring the distance between closest objects to be less than 4 pixels (910 nm). Ten movies were acquired with 1,000 frames each, tracking more than 10^6 objects. Only objects that remained on the surface for longer than 1 s were selected for analysis so that the lag time between initial and final observations of object position could be varied over one order of magnitude without eliminating objects from the distribution. This left $\sim 1,000$ objects that contributed $>50,000$ diffusive steps to the distribution at $\Delta t = 50$ ms.

The lag time was varied ($0.050 \text{ s} \leq \Delta t \leq 0.500 \text{ s}$) at constant t_{ac} by observing an object's position in frames j and $j + n$, where $n = \Delta t/t_{ac}$. The distribution of squared displacements was analyzed by first sorting the squared-displacement (r_k^2) data in ascending order and ranking each data point. Next, the cumulated (a.k.a. integrated) squared-displacement distribution (CSDD) was calculated as follows, where M is the number of observed displacements:

$$C(r_k^2, \Delta t) = 1 - k/M. \quad (5)$$

The CSDD is preferred to the raw squared-displacement distribution because it can be calculated directly from experimental data, without the need to choose an arbitrary bin width. This experimental CSDD was then fit using the integrated version of equation (1) with three modes (i.e., $N = 3$):

$$C(r^2, \Delta t) = \sum_{i=1}^N f_i \exp\left(-\frac{r^2}{4D_{i,app}\Delta t}\right). \quad (6)$$

Given the discussion of the previous section, therefore, the diffusion coefficients determined in this step should be interpreted as apparent rather than true diffusion coefficients. An experimental CSDD with $\Delta t = 0.1$ s is shown in Figure 1, along with the fit from equation (6). On this log-linear plot, a CSDD with a single diffusive mode would appear as a straight line with slope equivalent to $-1/D$; this is clearly not the case for this multimodal system. Apparent diffusion coefficients, $D_{i,app}$, that were derived from these fits are shown in Figure 2, where the apparent diffusion coefficient of each mode is plotted as a function of Δt^{-1} . Linear fits to each dataset are shown as dashed lines in which each intercept yields D_i and each slope yields σ_i^2 , and these parameters are shown in Table 1.

The fact that σ_i depends strongly on the diffusive mode reflects contributions to positional uncertainty from dynamic errors (Savin & Doyle, 2005). Recent theoretical treatment of this situation (Deschout et al., 2012) has shown that σ_i should obey equation (7) in which σ_S is the static error.

$$\sigma_i^2 = \sigma_S^2 + \frac{1}{3} D_i t_{ac}. \quad (7)$$

Ideally the static error is constant for a given set of experimental conditions, as is the case here. If equation (7) is used to extract σ_S from the data in Table 1, this yields $\sigma_S = 40$ (12), 62(5), 57(3) nm for modes 1, 2, and 3, respectively. Although these values vary somewhat, there is sufficient uncertainty that each is consistent with a value of $\sigma_S \approx 50$ nm. In fact, if the data in Figure 2 are refit with equation (7), substituted into equation (4), and the value of σ_S is required to be constant, the resulting fit is of similar quality ($R^2 = 0.9995$) but with $\sigma_S = 51.0(6)$ nm. This scheme also yields *true* diffusion coefficients of $D = 0.661(3)$, $0.169(4)$, $0.019(4)$ $\mu\text{m}^2/\text{s}$, for modes 1, 2, and 3, respectively. For comparison, the apparent diffusion coefficients when determined at $\Delta t = 0.05$ s gave $D = 0.92(3)$, $0.29(2)$, and $0.08(1)$ $\mu\text{m}^2/\text{s}$, for modes 1, 2, and 3, respectively. The fact that these values significantly overestimate the true values underscores the need to correct for positional uncertainty in both fast and slow diffusive modes.

Our estimated static error of 51 nm is less than the point spread function size of ~ 250 nm due to the accumulation of multiple photons from each object that permits a centroid of intensity calculation (Thompson et al., 2002). It has been shown that the positional uncertainty can be decreased to values less than ~ 20 nm using a Gaussian fit to determine object position (Wu et al., 2008), and the approach shown here is equally applicable to data obtained under those conditions. However, the added computational expense over a centroid of intensity calculation was not practical for high-throughput tracking methods used in the present work.

The differences between σ_S and σ indicate that both static and dynamic errors must be considered in multimodal diffusive processes. Dynamic error plays a large role in the apparent positional uncertainty of the faster mode 1, for example. In the extremely slow mode 3, static error is the dominant contribution. This result is certainly not surprising, but despite extensive literature on the subject, positional uncertainty is rarely accounted for in mobile objects exhibiting multimodal Brownian diffusion. The analysis presented here provides a simple way to correct for positional uncertainty due to both static and dynamic errors. If reliable data can be extracted on multiple diffusive modes, the instrumental uncertainty may also be estimated without the need for immobile objects. In cases where one does not have the original data and therefore cannot vary Δt to perform this analysis, it is sufficient to subtract a constant of $\sigma_i^2/\Delta t$, where σ_i is given by equation (7) and σ_S is known.

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Figure 1.

Representative CSDD for Fg adsorbed on TMS-coated fused silica. The experimental CSDD, calculated from equation (5) for $\Delta t = 0.1$ s, is shown as a function of $r^2/(4\Delta t)$ on log-linear axes. The solid line represents a 3-mode fit to these data using equation (6). Error bars represent 68% confidence intervals determined using Poisson statistics for the number of observations contributing to each data point.

Figure 2.

Dependence of the apparent diffusion coefficient on lag time. Three distinct modes of Fg diffusion on TMS were observed. The individual diffusion coefficients are shown as a function of inverse lag time, with a broken vertical axis to better illustrate the difference in slope between each dataset. The dependence of apparent diffusion coefficient on lag time, modeled by equation (4), is shown as a dashed line for each dataset. R^2 values for each fit are 0.9998, 0.9992, and 0.9951 for modes 1, 2, and 3, respectively.

Table 1

Parameters Used to Fit the Dependence of Apparent Diffusion Coefficients, $D_{i, app}$, on Lag Time. [★]

Mode	D ($\mu\text{m}^2/\text{s}$)	σ (nm)
1	0.671(5)	113(4)
2	0.159(3)	81(4)
3	0.016(2)	57(3)

[★]The number in parentheses represents uncertainty in the least significant digit as given by the standard error.