Measuring molecular motions inside single cells with improved analysis of single-particle trajectories

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Abstract

Single-molecule super-resolution imaging and tracking can measure molecular motions inside living cells on the scale of the molecules themselves. Diffusion in biological systems commonly exhibits multiple modes of motion, which can be effectively quantified by fitting the cumulative probability distribution of the squared step sizes in a two-step fitting process. Here we combine this two-step fit into a single least-squares minimization; this new method vastly reduces the total number of fitting parameters and increases the precision with which diffusion may be measured. We demonstrate this Global Fit approach on a simulated two-component system as well as on a mixture of diffusing 80 nm and 200 nm gold spheres to show improvements in fitting robustness and localization precision compared to the traditional Local Fit algorithm.

Introduction

Subcellular dynamics vary in time and over small size scales due to spatial and temporal variations such as transient interactions with molecular partners,[1] crowding by the nucleoid in bacteria,[2] or the presence of different lipid domains in membranes.[3] To measure the motions of biological molecules such as proteins and lipids in the face of this complicated local environment, single-molecule super-resolution fluorescence microscopy data must be analyzed with a method that systematically accounts for heterogeneity. One approach to single-particle tracking (SPT) derives the apparent diffusion coefficient from each individual trajectory from the mean square displacement (MSD) vs. time lag curve.[4–6] The trajectory of each particle is thus assigned an average diffusion coefficient with this MSD approach, and heterogeneous diffusion is described by dividing the collection of MSD curves into diffusive populations. The number of trajectories in each population of curves may be taken as an estimate of the relative proportions of these diffusive populations.[7–9] However, in real systems, heterogeneous diffusion can be observed even over the course of the trajectory of a single molecule, and this single-track MSD analysis specifically disallows the case where a single molecular trajectory experiences multiple diffusive modes by providing only the average diffusion coefficient for each track.

An approach that accounts explicitly for such heterogeneous motion considers the entire collection of single-molecule steps instead of dividing these steps into individual tracks. This collection of step data can then be quantified based on the cumulative probability distribution (CPD) of the collection of squared step sizes to explicitly account for spatial and temporal heterogeneities, and increase the signal-to-noise ratio. Single-step CPD analysis is therefore a diffusion estimation technique that has had impact across fields by characterizing diverse biological systems such as artificial membranes, leukocytes, bacterial membranes, neurons and artificial materials.[10–22]

Alternatively, a number of Bayesian[23–26] and machine learning algorithms[27] can be used to estimate the number of diffusive components and measure their properties, but the complexity of these methods poses a significant barrier to intuitive understanding of the underlying modes of heterogeneous motion. Fluorescence correlation spectroscopy (FCS) and the related methods of spatiotemporal image correlation spectroscopy (STICS), raster image correlation spectroscopy

(RICS), or particle image correlation spectroscopy (PICS) can also quantify diffusion; these approaches all employ spatial or temporal correlation functions which can also be fit to multi-component diffusion models,[28–32] but it is rare for the signal-to-noise to be high enough for the analysis of complex heterogeneous motion such as are found in bacterial systems.[33] The ability of single-particle tracking to isolate high quality trajectories from noisy single molecule data can present a more attractive conduit for analysis.

Despite its advantages, the commonly employed CPD analysis method involves a two-step fitting process where the CPD and MSD curves are fit sequentially.[11,13] We present here an approach that combines this two-step Local Fit process into a single-step multi-domain Global Fit algorithm. We analyze simulated trajectories of multiple diffusive components to measure the improvements in the diffusion coefficient estimation error and find that Global Fit is superior to the traditional Local Fit CPD analysis algorithm. We then consider the diffusion of 80 nm and 200 nm gold spheres in glycerol water solution to show that Global Fit outperforms Local Fit in a real system. We report improvements in precision, robustness, and simplicity of use.

Materials and Methods

Imaging and Tracking.

Slides were imaged at room temperature using wide-field epifluorescence microscopy in an Olympus IX71 inverted microscope with a 100 $\times$, 1.40 NA oil immersion objective (in Zeiss Immersol 518F immersion oil) and appropriate excitation, emission, and dichroic filters (Semrock LL01-488, Semrock BLP01-488 and Semrock Di01-R488, respectively). After a 3 $\times$ beam expander, a Photometrics Evolve EMCCD camera with > 90% quantum efficiency captured the images at 100 frames per second. Each camera pixel corresponds to a 49 nm $\times$ 49 nm area of the sample. The gold spheres were illuminated with a 488 nm laser (Coherent Sapphire 488-50), that was circularly polarized with a quarter waveplate (Tower Optical AO15Z 1/4). Single molecule positions were associated into tracks with the Hungarian algorithm[34] according to an exponential merit function.[35]

Diffusion of gold spheres in glycerol.

Gold nanoparticles with diameters 80 and 200 nm (BBI Solutions) were dispersed in 50% glycerol. 5 μ L of the mixture was sandwiched between two glass coverslips. The second through fifth time lags were used for both the Global Fit and Local Fit algorithms to reduce the magnitude of the fitting residuals. Unweighted least squares fitting was performed with the Matlab built-in function *lsqnonlin*.

Simulations.

Diffusion was simulated by generating 10^3 steps from a zero-mean normal distribution with variance equal to $2Dt_{\text{frame}}$, where D is the desired diffusion coefficient and t_{frame} is the simulated camera exposure time which was set to 0.04 seconds. Localization precision was simulated by adding zero-mean Gaussian-distributed random numbers to the simulated trajectories; the localization precision, or the standard deviation of the random numbers, was varied from 4.9 nm to 73.5 nm. Each simulation was repeated 10^3 times. The first 10 time lags were used for both the Global Fit and Local Fit algorithms and unweighted least squares was performed with the Matlab built-in function *lsqnonlin*.

Bootstrapping.

For the analysis of the tracks of gold spheres, histograms of estimated diffusion coefficients and population weights were produced by bootstrapping the fitting procedure. The total set of 13232 squared step sizes was sampled with replacement 300 times to produce 300 unique data sets each with as many values as the original data set. These bootstrapped data sets were then fit with either the Global Fit or Local Fit method.

CPD Local Fit.

To probe heterogeneous diffusion, the cumulative probability distribution (CPD) of squared step sizes (Δr^2) was calculated from the tracks of diffusing molecules at each time lag (τ) between frames in the trajectory. There is one CPD curve, CPD_i , for each time lag considered, and each

CPD_i was fit to the multi-term exponential fit[11] with the appropriate number of terms (three terms shown here for instance):

$$CPD_i = 1 - \alpha_1 \times \exp\left(\frac{-\Delta r_i^2}{MSD_{1,i}}\right) - \alpha_2 \times \exp\left(\frac{-\Delta r_i^2}{MSD_{2,i}}\right) - (1 - \alpha_1 - \alpha_2) \times \exp\left(\frac{-\Delta r_i^2}{MSD_{3,i}}\right) \quad (1)$$

This series of fits, where i , runs from 1 to the number of time lags considered, N_τ , estimates three mean squared displacements, MSD_1 , MSD_2 , and MSD_3 , as a function of time lag for $N_D = 3$ diffusive populations with weights α_1 , α_2 , and $(1 - \alpha_1 - \alpha_2)$, respectively. Each of the three MSD curves is then fit to a model—here of 2D unconfined diffusion—to extract the diffusion coefficient of the respective population of molecules. For example, for population 1, the second-step fitting function is:

$$MSD_1 = 4D_1\tau + 4\sigma_1^2, \quad (2)$$

where D_1 is the diffusion coefficient of population α_1 , τ is the domain of time lags and σ_1 is the localization precision for population α_1 . If one uses the first 5 time lags to estimate the diffusion coefficients of three populations, the total number of fitting parameters in this local CPD fitting approach is: $5 \times N_\tau + 2 \times N_D = 31$ here with $N_\tau = 5$, and $N_D = 3$.

CPD Global Fit.

Instead of fitting in separate steps, the set of empirical CPDs may be fit all at once by incorporating the MSD functions (Equation (2)) into Equation (1). Conceptually, this can be understood as the sharing of redundant parameters, such as the weight of population 1, i.e., α_1 .

The free parameters in the combined fitting function now include only N_D diffusion coefficients (one for each population), a single localization precision, σ , shared among all populations, and all but one of the population weights because one is estimated using the others. For instance, if one wishes to estimate the diffusion coefficients of three populations, the total number of fitting parameters is 6 (three diffusion coefficients, one localization precision parameter, and two

population weight parameters). This number of fitting parameters in Global Fit is hugely improved from the 31 parameters necessary for Local Fit.

We implemented Global Fit with a Matlab-specific formulation that exchanges several nonlinear least squares problems for a single larger nonlinear least squares problem. See <https://github.com/BiteenMatlab/SingleMoleculeDataAnalysis> for complete code; the key snippets of our code describing the use of this algorithm for a three-population Global Fit are given here:

```
msdFun = @(tau,p)cat(2, 4*p(1)*tau + p(2), 4*p(3)*tau + p(2), 4*p(4)*tau +
    p(2));
cpdFun = @(x,y,p)1-p(5)*exp(-x/y(1))-p(6)*exp(-x/y(2))-(1-p(5)-p(6))*exp(-
    x/y(3));
linCell = @(x)cat(1,x{:});
fHandle = @(p,tau,sqSteps,ranks)linCell(cellfun(@(x,y)x-
    y,cellfun(@(x,y)cpdFun(x,y,p),sqSteps,num2cell(msdFun(tau,p),2),'uniformoutput',0),ranks,'uniformoutput',0));
[FP,~,resids] = lsqnonlin(@(p)fHandle(p,t,sqSteps,ranks),pStart, LB, UB);
```

In the above code, $pStart$ is the vector of starting parameters (1×6 in this case of three unconfined diffusive components). $sqSteps$ is a $1 \times N_\tau$ cell array of the sorted lists of squared step sizes for each time lag, and $ranks$ is a $1 \times N_\tau$ cell array with each element containing values from 0 to 1 having the same size as the corresponding element from the $sqSteps$ cell array. LB and UB are the lower and upper bounds for the parameters found in $pStart$.

Results and Discussion.

To illustrate the power of CPD Global Fit, we implemented the algorithm in Matlab (Methods) and applied this analysis to simulated diffusive 2D trajectories (Figure 1A) with a combination of diffusion coefficients ($D_1 = 1 \mu\text{m}^2\text{s}^{-1}$ and $D_2 = 0.01 \mu\text{m}^2\text{s}^{-1}$). For each trajectory, the collection of squared displacements given a certain time lag are used to compute the empirical cumulative probability distribution for that time lag. Figure 1B compares the Global Fit method to the traditional Local Fit method. In both methods, the CPD of squared step sizes is computed at each of the first 10 time lags (black and grey curves). The Local Fit method (green arrows) first fits

each of the CPD curves independently to estimate the mean squared displacements (MSDs) of the two diffusive populations for each curve as well as to provide an average estimate of the population weights, α_1 and $(1 - \alpha_1)$. The two resulting MSD vs. time lag curves (blue and orange) are then fit in a second step to estimate the diffusion coefficients of each of the two populations. Global Fit, on the other hand, combines all of these fitting operations into a single least squares minimization step (blue arrow). This Global Fit process greatly reduces the total number of fitting parameters. In Figure 1B, Local Fit requires 3 free parameters ($\text{MSD}_{1,i}$, $\text{MSD}_{2,i}$ and $\alpha_{1,i}$) for each CPD curve fit and 2 for each MSD curve (D and the localization precision, σ) for a total of 34 parameters. Global Fit, which does not separate the CPD and MSD fits, in this case uses only 4 fit parameters (D_1 , D_2 , α_1 , and σ).

Based on the analysis of simulations of unconfined 2D diffusion of two diffusive populations as in Figure 1A, Figure 2 describes the expected behavior of the Global Fit and Local Fit methods. At each condition, 10^4 trajectories of 10^3 steps were randomly generated with localization precision, σ (Methods). Figure 2A shows the standard deviation (S.D.) of the diffusion coefficient estimates for trajectories simulated with σ that varies from 4.9 nm to 73.5 nm. At all values of σ , Global Fit (solid lines) outperforms Local Fit (dotted lines) for both diffusive components (blue and orange). Notably, once σ approaches 70 nm (black arrow in Figure 2A), Local Fit completely fails to estimate the diffusion coefficient of the faster diffusive population. This breakdown is caused when numerical instability due to poor data quality produces mostly meaningless diffusion coefficient estimates. The increased numerical stability of the Global Fit algorithm enables a precise and unbiased estimation of the diffusion coefficients even in the presence of 4 diffusive populations; the Local Fit algorithm fails completely to measure motion in this situation (Supporting Information Figure S1). Overall, by constraining all CPD curves to the same diffusion model parameters, Global Fit is more robust to increased complexity and decreased data quality. The histograms in Figures 2B and 2C present the results of the simulations used in Figure 2A at $\sigma = 4.9$ nm and 38 nm. For this two-population simulation, at both low and high levels of localization precision, the diffusion coefficients of both populations are measured without bias by both fitting methods. However, the decreased standard deviations for diffusion coefficient estimations indicate that the results from Global Fit (solid curves) are

more rigidly centered on their mean value. In other words, sampling error is less likely to skew the result of Global Fit when a two-term CPD model was used.

The Global Fit algorithm also shows improved performance in applications to experimental measurements where the ground truth is not known. We imaged suspensions of spherical gold nanoparticles of diameter 80 nm or 200 nm dispersed in 50% glycerol (Methods) and tracked the particles as shown in Figure 3ABC. Figure 3D reports the bootstrapped results of Global Fit and Local Fit of a one-population diffusion model to movies of samples containing only 200 nm gold spheres. Global Fit results in a histogram of diffusion coefficient estimates with mean $0.24 \mu\text{m}^2\text{s}^{-1}$ and standard deviation $0.0061 \mu\text{m}^2\text{s}^{-1}$, whereas local fitting of the same data set estimates a larger diffusion coefficient of $0.27 \mu\text{m}^2\text{s}^{-1}$ and a smaller standard deviation of $0.0055 \mu\text{m}^2\text{s}^{-1}$. Figure 3E shows the estimated diffusion coefficients of 80 nm gold spheres in 50% glycerol. Again, the mean diffusion coefficient estimate increases and standard deviation decreases when Local Fit is used instead of Global Fit. Here, the diffusion coefficient increases from $0.74 \mu\text{m}^2\text{s}^{-1}$ to $0.82 \mu\text{m}^2\text{s}^{-1}$ and the standard deviation decreases from $0.026 \mu\text{m}^2\text{s}^{-1}$ to $0.022 \mu\text{m}^2\text{s}^{-1}$ when comparing the Global Fit and Local Fit methods, respectively. See Supporting Information Table S2 for these results in table form. In this case of single-component diffusion, we found that Local Fit resulted in a more precise measurement of the diffusion coefficient. Importantly, this increased precision is likely due to the smaller fitting residuals that arise from having an increased number of fitting parameters in Local Fit; the increased precision should not be taken to imply an increase in accuracy, because there are no guarantees that the minima found in least squares minimization is the global minimum.

Single-particle tracks within mixtures of both nanoparticle sizes require a two-population diffusion model. Here, Global Fit more precisely estimates the diffusion coefficients. Figure 3F shows histograms of the diffusion coefficient estimates for this scenario, where Global Fit estimates the two diffusion coefficients to be $0.33 \mu\text{m}^2\text{s}^{-1} \pm 0.0074 \mu\text{m}^2\text{s}^{-1}$ and $0.76 \mu\text{m}^2\text{s}^{-1} \pm 0.047 \mu\text{m}^2\text{s}^{-1}$ corresponding to the 200 nm and 80 nm gold spheres, respectively. Local Fit, on the other hand, estimates the diffusion coefficients to be $0.37 \mu\text{m}^2\text{s}^{-1} \pm 0.021 \mu\text{m}^2\text{s}^{-1}$ and $0.98 \mu\text{m}^2\text{s}^{-1} \pm 0.15 \mu\text{m}^2\text{s}^{-1}$. See Supporting Information Table S2 for these results in table form. In this scenario where multiple diffusive populations are present, the improved parameter rigidity of Global Fit is readily apparent. As the complexity of the model increases, Global Fit offers a more

robust method for estimating diffusion coefficients. It should be noted, however, that both Global Fit and Local Fit algorithms performed equally well in the estimation of the population weights, α and $(1 - \alpha)$ (Supporting Information Figure S2).

Alternatively, we investigated the diffusion of single DiI fluorescent molecules in supported lipid bilayers (methods in Supporting Information Figure S3), and uncovered sample inhomogeneities that are outside of the scope of this work. Supporting Information Figure S4 compares the Local Fit and Global Fit methods with one and two-term fits to DiI trajectories and demonstrates the presence of two diffusive populations. DiI molecules in a homogeneous lipid environment should diffuse at around $2 \mu\text{m}^2\text{s}^{-1}$, and indeed, the faster population has $D_1 = 2.5 \mu\text{m}^2\text{s}^{-1}$, which is near to this value (Supporting Information Figure S5). However, the analysis also reveals a slower diffusive population ($D_2 = 0.4 \mu\text{m}^2\text{s}^{-1}$; Supporting Information Table S1), which is expected due to interactions between molecules of DiI and the glass surface and defects in the lipid bilayer. Global Fit outperformed Local Fit in terms of diffusion coefficient estimation precision and observe that the freely diffusing DiI accounts for 50% of the diffusing molecules as shown in Supporting Information Figure S5.

Conclusions.

In this work, we introduced global CPD fitting, a straightforward method to enhance the precision and simplicity of a commonly used diffusion coefficient estimation technique by combining redundant parameters. Instead of estimating the mean squared displacements of diffusing particles only to then fit those results in a second curve fitting step (Figure 1B), we presented a multi-domain fit that accomplishes both steps at once. The resulting reduction in degrees of freedom of the fitting operation increases the estimation precision and robustness by increasing the rigidity of the fitting parameters. The enhanced precision and robustness of the Global Fit algorithm introduced here enables precise, quantitative investigations of systems with increased complexity, for instance motion that includes more than one or two diffusive components. This Global Fit method is not restricted to freely diffusing molecules, and can be extended to any type of diffusion such as confined diffusion, diffusion with flow, or anomalous diffusion.

Artwork and Tables with Captions:

Figure 1. Schematic diagram of Local Fit and Global Fit. (A) An example pair of diffusive 2D trajectories with 10^3 steps each and diffusion coefficients of $1 \mu\text{m}^2\text{s}^{-1}$ and $0.01 \mu\text{m}^2\text{s}^{-1}$ (blue and orange trajectories, respectively). The cumulative probability distribution (CPD) approach analyzes data from trajectories like these to extract the diffusion coefficients, D_1 and D_2 , and relative weights, α_1 and $\alpha_2 = (1 - \alpha_1)$, of each diffusive population. (B) The CPD of squared step sizes is computed at each of the first 10 time lags. The resulting curves are plotted here with decreasing intensity from the first time lag (black) to the tenth (light gray). For Local Fit (green arrows), first each CPD is fit to a two-population model yielding two series of mean squared displacement (MSD) values and a population weighting factor, α_1 , that is the average from all the fits. The color of each resulting MSD vs. time lag curve (bottom left) indicates the color of the corresponding trajectory in (A). Next, the two MSD curves are fit separately to yield D_1 and D_2 . Instead, for Global Fit (blue arrow), all 10 CPD curves are fit at once to yield α_1 , D_1 and D_2 . Local Fit here requires a total of 34 fitting parameters whereas Global Fit requires only 4 fitting parameters (two diffusion coefficients, a population weight, and a localization precision).

Figure 2. (A) Standard deviation of diffusion coefficient estimations for simulations of unconfined 2D diffusion of two diffusive populations. Trajectories were simulated as in Figure 1A with 10^3 time steps for each of two diffusion coefficients, $0.1 \mu\text{m}^2\text{s}^{-1}$ and $0.01 \mu\text{m}^2\text{s}^{-1}$, and white noise was added to simulate a range of localization precisions. The trajectories of both diffusive populations at each noise level were combined then analyzed with the CPD Global Fit (blue) and Local Fit (orange) methods using a two-population model (Equation 1 with only the first two terms). 10^4 trajectories were randomly generated and each was analyzed; the standard deviations of the estimated diffusion coefficients, D_1 and D_2 , are shown. The black arrow corresponds to the point at which numerical instability due to poor data quality causes the Local Fit algorithm to fail to estimate the faster of the two diffusion coefficients. (B) and (C) Histograms of the estimated diffusion coefficients in (A) at localization precisions of 4.9 nm and 38 nm, respectively.

Figure 3. (A), (B), (C) 40 tracks of 200 nm gold spheres, 13 tracks of 80 nm gold spheres, and 28 tracks of a mixture of both sizes of gold spheres, all diffusing in 50% glycerol, respectively. (D) Histograms of the estimated diffusion coefficient for 200 nm gold spheres in 50% glycerol in water solution from fits to a one-component model with the Global Fit (solid line) and Local Fit (dashed line) methods. (E) Histograms of the estimated diffusion coefficient for 80 nm gold spheres under the same conditions. (F) Estimated diffusion coefficients of a mixture of 200 nm and 80 nm gold spheres in a 50% glycerol in water solution fit using a two-component model by Global Fit (solid lines) and Local Fit (dashed lines). The blue and orange line colors correspond to the sphere sizes in panels ‘D’ and ‘E’, respectively.

Author Contributions

DR and JB designed the research. DR developed the Global Fit algorithm, performed the simulations, carried out the experimental measurements, and analyzed the data. DR and JB discussed all the results. DR wrote the paper, which was edited by both authors. Both authors read and approved the final manuscript.

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Graphical Abstract