Implementing Numerical Fluorescence Correlation Spectroscopy Under Non-Standard Measurement Conditions to Accurately Assess Molecular Dynamics

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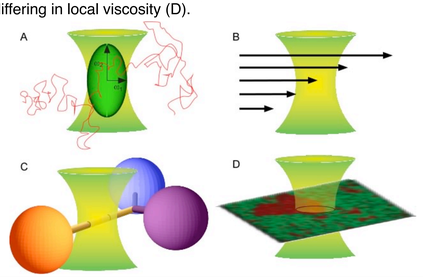
Abstract

Some measurement scenarios present particular challenges to Fluorescence Correlation Spectroscopy (FCS) since they involve complex interactions of fluorescent tracers and the confocal detection volume that cannot be modeled analytically. We describe a novel numerical approach to FCS that circumvents the limitations of conventional analytical models, enabling meaningful analyses even under extraordinarily unusual measurement conditions. Numerical Fluorescence Correlation Spectroscopy (NFCS) involves quantitatively matching experimental correlation curves with synthetic curves generated via diffusion simulation or direct calculation based on an experimentally determined 3D map of the detection volume. Model parameters are adjusted iteratively to minimize the residual differences between synthetic and experimental correlation curves. In order to reduce analysis time, we distribute calculations across a network of processors. As an example of the effectiveness this new approach, we demonstrate that synthetic autocorrelation curves correspond well with experimental data and that NFCS diffusion measurements of Rhodamine B remain constant, despite distortion in a confocal detection volume.

Motivation

Over the last thirty years, Fluorescence Correlation Spectroscopy has grown to a place of prominence in microscopy circles as a versatile method of analyzing molecular dynamics. In traditional one-photon FCS, a sample with a fluorescent tracer is placed in a focused laser beam, and the fluorescent bursts are captured and recorded as the tracer moves through the detection region (A). This fluorescence signal is autocorrelated and fit with an analytical expression to extract a quantity of interest, such as diffusion constant, triplet-state dynamics, reaction kinetics, or rate of bulk flow. FCS has achieved the capability of extracting this information even from samples of single-molecule concentration.

Although analytical expressions exist for a good variety of experimental conditions, some scenarios present particular challenges to deriving a mathematical expression for the interaction of tracers and the three-dimensional detection volume, especially if they involve some nanoscale geometric constraint that limits the tracers' freedom in the detection region, such as diffusion in a gradient flow profile (B), diffusion in or on a nanotubule (C), or diffusion in a two-component lipid membrane with microdomains differing in local viscosity (D).



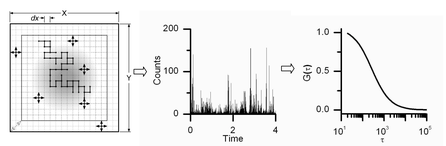
Numerical Fluorescence Correlation Spectroscopy

The autocorrelation curves produced under these kinds of conditions still contain valuable information, even though it can't be accessed via traditional FCS. Rather, the complex interactions between fluorescent tracers and the detection volume in many of these cases can be simulated quite easily or modeled by other numerical methods unamenable to standard curve-fitting. We therefore propose an extension method of FCS, dubbed Numerical Fluorescence Correlation Spectroscopy (NFCS) that circumvents the analytical expression of traditional FCS by making use of numerical techniques for modeling the behavior of fluorescent molecules in the detection region.

Instead of fitting the autocorrelated data with an analytical expression, the new method compares the experimental data with a synthetic curve based on a guess of the parameters of interest. These parameters are then adjusted iteratively to minimize the differences between the experimental and synthetic curves, as represented in the sum of squared residuals. The synthetic curves may be obtained alternatively via simulation or direct calculation based on numerical models of the detection volume and molecular motion.

Simulation

Our single-molecule diffusion simulator superimposes onto a numerical map of the detection profile a 3D lattice along which molecules walk. The step size along the lattice, *dx*, is adjusted per species to equal to the root-mean-squared diffusion distance given the species' diffusion constant and a step time, typically on the order of 500 ns. Each step, the molecule moves one lattice position in a random direction, and the fluorescence probability is calculated for a molecule in that position. The fluorescence probabilities are summed over all molecules for a given bin width, and the final simulated collected fluorescence emission is sampled from a Poisson distribution, giving a real-time record of fluorescence bursts. This record is then autocorrelated for NFCS analysis.



To render extensive simulations for analysis more feasible, we divide each simulation in time segments and distribute the segments across a bank of user work-stations for processing in parallel. The simulations run at a low priority, allowing them to recover otherwise-wasted CPU cycles without disrupting other users. The simulator makes use of a load-balancing procedure to maximize performance across a diverse network and can thus accommodate both dedicated scientific computing networks and the collections of laboratory workstations available in most academic settings. We have also developed a novel distributed autocorrelation algorithm that begins the autocorrelation at the remote data source to reduce the amount of data transferred across the network.

Direct Numerical Calculation

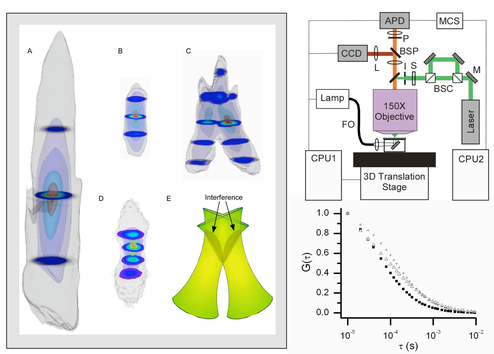
The alternative method to simulation for generating synthetic curves in NFCS is direct calculation from numerical models of both the detection volume profile and molecular motion. This method is particularly useful for molecules that obey standard Brownian motion in abnormally shaped detection volumes. The autocorrelation function is written as the probability of producing a photon at two different locations, moderated by the probability of moving from the first location to the second in the given lag time, integrated over all space:

where *O* is the normalized detection volume profile and *D* is the diffusion constant. The double integral can be calculated efficiently with a convolution and summation.

Experimental Validation

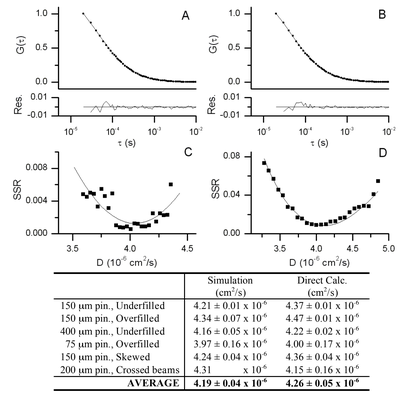
As an example of the utility and effectiveness of NFCS, we applied the method to analyze diffusion data for Rhodamine B using the 543 line of a 5 mW HeNe laser under a variety of optical conditions, altering the detection region geometry with different combinations of pinhole size and objective back-aperture filling. For demonstration purposes, we also created a highly irregular detection volume using two slightly misaligned laser beams (C). Near the focal plane, the two beams cross to form a profile with varied structure due to laser interference (E). For each scenario, the confocal detection volume was mapped by scanning a 170-nm bead through the detection region.

Differently shaped volumes produced autocorrelation curves not only with longer or shorter crossing times, but also with different contours, depending on the different geometric features of the profiles. These difference indicate deviations in the detection volume from traditional FCS theory, which are accommodated in NFCS by the numerical map of the detection volume profile.



NFCS Analysis and Results

The autocorrelation function of the Rhodamine B diffusion data for each detection profile was analyzed with NFCS via both simulation (A) and direct calculation (B). Simulation (D, points) and calculation (D, line) produced similar autocorrelation curves. We use the Simplex algorithm to minimize the sum of squared residuals. Since simulated data are subject to stochastic noise, the merit function has a large number of local minima which makes getting a precise value difficult (C). For NFCS via simulation, we typically use the Simplex algorithm to estimate the diffusion constant and then fit a range of values near the estimate with a quadratic to find the actual minimum.



	Simulation (cm^2/s)	Direct Calc. (cm^2/s)
150 μm pin., Underfilled	$4.21 \pm 0.01 \times 10^{-6}$	$4.37 \pm 0.01 \times 10^{-6}$
150 μm pin., Overfilled	$4.34 \pm 0.07 \times 10^{-6}$	$4.47 \pm 0.01 \times 10^{-6}$
400 μm pin., Underfilled	$4.16 \pm 0.05 \times 10^{-6}$	$4.22 \pm 0.02 \times 10^{-6}$
75 μm pin., Overfilled	$3.97 \pm 0.16 \times 10^{-6}$	$4.00 \pm 0.17 \times 10^{-6}$
150 μm pin., Skewed	$4.24 \pm 0.04 \times 10^{-6}$	$4.36 \pm 0.04 \times 10^{-6}$
200 μm pin., Crossed beams	4.31×10^{-6}	$4.15 \pm 0.16 \times 10^{-6}$
AVERAGE	$\mathbf{4.19 \pm 0.04 \times 10^{-6}}$	$\mathbf{4.26 \pm 0.05 \times 10^{-6}}$

Conclusion

We described a new method for the analysis of molecular dynamics that is based on traditional Fluorescence Correlation Spectroscopy, but circumvents many of its inherent limitations by numerically modeling the interaction of fluorescent molecules and the detection volume. Direct calculation of the autocorrelation function is generally faster than simulation and less prone to random variation, but simulation allows greater freedom to model a variety of scenarios that cannot be described analytically. We believe that NFCS will be generally applicable whenever experimental conditions deviate from the assumptions of traditional FCS, including situations with nanoscale geometric constraints. Thus, we anticipate that the numerical concepts described here will broaden the possibilities for molecular dynamics analyses in many new and interesting systems.

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