Enhanced Resolution and Sensitivity in Fluorescence Fluctuation Measurements Using Multi-Modal Data Acquisition and Global Analysis

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ABSTRACT

Fluorescence correlation spectroscopy (FCS) and related fluctuation spectroscopy and microscopy methods have become important research tools that enable detailed investigations of the chemical and physical properties of molecules and molecular systems in a variety of complex environments. When analyzed successfully fluctuation measurements often provide unique information that is otherwise difficult to measure, such as molecular concentrations and interaction stoichiometry. However, information recovery *via* curve fitting of fluctuation data can present challenges due to limited resolution and/or problems with fitting model verification. We discuss a new approach to fluctuation data analysis coupling multi-modal fluorescence measurements and global analysis, and demonstrate how this approach can provide enhanced sensitivity and resolution in fluctuation measurements. We illustrate the approach using a combination of FCS and fluorescence lifetime measurements, here called τFCS, and demonstrate the capability to recover the concentration of two independent molecular species in a two component mixture even when the species have identical diffusion coefficients and molecular brightness values. This work was partially supported by NSF grants MCB0817966 and DMR0907435.

Keywords: Fluorescence Correlation Spectroscopy, FCS, Fluorescence Lifetime, TCSPC, Global Analysis, τFCS.

1. INTRODUCTION

Fluorescence correlation spectroscopy (FCS) has become an important research tool that enables detailed investigations of the chemical and physical properties of molecules or molecular systems in a variety of complex environments [1-7]. When analyzed successfully fluctuation measurements often provide unique information that is otherwise difficult to measure, such as molecular concentrations and interaction stoichiometry. However, information recovery *via* curve fitting of fluctuation data can present challenges due to limited resolution and/or problems with fitting model verification. This work demonstrates a new conceptual approach for acquisition and analysis of FCS data. Specifically, we apply simultaneous acquisition of FCS and fluorescence lifetime data and then use global analysis to analyze both data types with common linked fundamental parameters. We demonstrate that this approach provides dramatically enhanced resolution for FCS studies, demonstrating the capability to accurately determine the concentration of two independent molecular species with the same diffusion coefficient – a

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sample that standard FCS applications would be unable to resolve. We also discuss how this new analysis approach reduces problems associated with model discrimination during curve fitting procedures.

2. BACKGROUND

Fluorescence correlation spectroscopy and other fluctuation methods offer unique capabilities to measure dynamics over a wide range of time-scales as well as to accurately measure molecular concentrations and directly read-out the stoichiometric composition of interacting molecules due to the self-calibrating nature of fluctuation measurements [8]. FFS techniques have also proven to be remarkably useful for characterizing molecular dynamics and interactions within complex systems [9-12]. Information recovery from FCS requires curve fitting of fluctuation signals to different physical models. When accurate models are used, impressive resolution of sample composition is often achievable. However, there are two major challenges that can limit the overall applicability of FCS and related methods. First, standard FCS measurements offer limited capability to discriminate between fitting models, and knowledge of the sample composition or physical dynamics driving fluctuations may not be available *a priori*, particularly within complex environments such as living cells. For example, it is often possible to fit FCS data to multiple physical models with comparable "goodness of fit" as determined by χ^2 values and analysis of residuals. An example of this problem is shown in Fig. 1, in which several different physical models all produce reasonable fits to data acquired from a sample containing molecules with two distinct diffusion coefficients. Second, FCS data has limited resolution such that it is not always possible to resolve all sample components of interest, even when accurate physical models are used for curve fitting. For example, FCS cannot resolve two molecules with identical diffusion coefficients, with reported resolution limits requiring differences in diffusion coefficients of at least 1.6 times [13]. Interactions between molecules of similar or identical molecular weight are thus not resolvable by diffusion analysis, a limitation that would be useful to overcome.

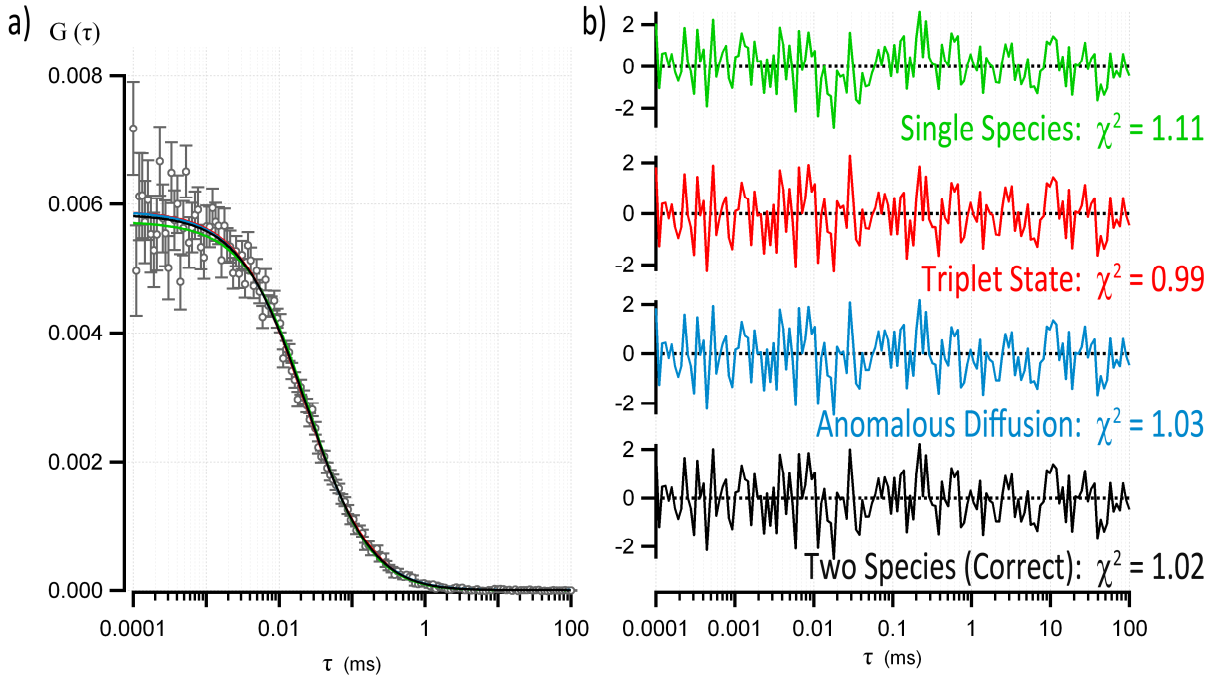

Figure 1 – Determining the correct underlying physical model using data fitting alone is a non-trivial task. Here, four different physical models have been fitted to a calculated autocorrelation function from a two-species sample (a) [from top to bottom: single diffusing species, single diffusing species with triplet state kinetics, single species anomalous diffusion, two species diffusion]. The returned residuals (b), and corresponding χ^2 goodness of fit parameters render the choice based on fit alone impossible, thus requiring additional *a priori* knowledge about the model in order to fit the data.

A variety of strategies have been implemented to overcome the resolution limits, including multi-color FCS measurements and the development of numerous molecular brightness based statistical analysis approaches [8, 14-20]. The use of fluorescence lifetime measurements has also been introduced as a way to distinguish between molecular species that are not resolvable based on diffusion or molecular brightness analyses [21]. Here, we also explore the possibility of using fluorescence lifetime measurements as a contrast enhancing variable, while still capturing the unique information available from FCS measurements, yet use a fundamentally different approach from previously reported analysis strategies [21, 22]. In particular, we explore the use of global fitting algorithms [23-28] to simultaneously fit both FCS and lifetime data, an approach we refer to as τFCS. Curve fitting using common global parameters 'linked' across multiple data sets (see Theory section) reduces the number of free fitting parameters and effectively constrains the fitting parameter space that can fit all experimental data simultaneously. Such constraints can greatly enhance model discrimination capabilities in curve fitting routines and also enhance resolution. We document that this approach is remarkably successful for resolving the physical properties of a two-component mixture even for the case where diffusion and molecular brightness analysis are unable to resolve the two species. We note that both lifetime and FCS have previously been combined using a software filter based analysis protocol, referred to as FLCS [21, 29]. Both FLCS and τFCS fundamentally transform the resolution limits

of FCS [13], although we find that the τFCS approach offers significant practical advantages and has significantly less demanding requirements on signal statistics than previously reported methods.

3. THEORY

Simultaneous global fitting of both lifetime and FCS data requires that the theory for each be written in terms of common linkable parameters. Fluorescence lifetime measurements require nanosecond time resolution and thus τFCS theory describes pulsed laser excitation, spans picosecond to second time scales, and incorporates effects due to saturation and finite fluorescence lifetimes [30-34]. This theory requires two time scales: the micro-time (ps to ns) is used to describe the excited state dynamics of fluorescent molecules and resets to zero after each laser pulse, while macro-time (μs to s) describes longer time scale fluorescence fluctuations due to physical or chemical dynamics. The theory presented below is for two-photon laser excitation. A detailed derivation of the following theory can be found in [35].

The average fluorescence signal from a single species, measured on the microscopy, can be written as:

$$\langle F \rangle = \psi \langle C \rangle V \tag{1}$$

The "molecular brightness" parameter, ψ, is so named because it reports the average number of fluorescence photons detected per molecule per second [8, 36, 37] from molecules within the measurement volume, V. The average concentration is written as $\langle C \rangle$. Using this same notation, the fluorescence lifetime data from a multi-component sample, acquired by time correlated single photon counting (TCSPC), can be written as:

$$F_{hist}^{data}(t') = \sum_i \frac{T \Delta t' \psi_i \langle C_i \rangle V}{\tau_i \left(1 - e^{-1/\tau_i f_p}\right)} e^{-t'/\tau_i} \tag{2}$$

Here τ_i is the lifetime of each molecular species, T the total data acquisition time, and $\Delta t'$ is the binning width for TCSPC acquisition under pulsed laser excitation with pulse repetition rate f_p.

Similarly, FCS theory for a multi-component sample is described as [2, 4, 38]:

$$G(\tau_{fcs}) = \frac{\gamma}{V} \frac{\sum_i \psi_i^2 \langle C_i \rangle A_i(\tau_{fcs})}{\left(\sum_i \psi_i \langle C_i \rangle\right)^2} \tag{3}$$

where $A_i(\tau_{fcs})$ represents the temporal relaxation of the correlation function for species i, and the γ-factor describes the steepness of the observation volume boundaries [34]. For pure diffusion (diffusion coefficient D) in a

three dimensional Gaussian volume with radial beam waist w_0, and an axial beam waist aw_0, the temporal relaxation is described by:

$$A\left(\tau_{fcs}\right)=\left(1-\frac{8D\tau_{fcs}}{w_0^2}\right)^{-1}\left(1-\frac{8D\tau_{fcs}}{a^2w_0^2}\right)^{-1/2} \tag{4}$$

Using these equations, we can simultaneously fit both fluorescence lifetime and FCS data sets using common global parameters across both data types. We note also that the two measurement types have different dependencies on the global parameters, i.e. the amplitude of the lifetime data scales with the product of the concentration and molecular brightness while the amplitude of the FCS data also includes the square of the molecular brightness multiplied by the concentration. These differences in parameter dependence provide significant constraints for model discrimination in fitting routines.

4. METHODS

We use simulated data sets to demonstrate that τFCS can accurately recover the concentrations, diffusion coefficients, molecular brightness values, and excited state lifetimes for binary mixtures with identical diffusion coefficients. FCS measurements alone would not detect two separate sample components for this same sample, illustrating the unique resolution enhancements introduced by using the multi-parameter fluorescence measurements with global fitting. Simulated data sets were created using Eqs. (2) & (3) for a given parameter set followed by the addition of random noise. TCSPC histograms were created with a total of 1×10^7 counts followed by the addition of Poissonian noise for a given number of counts per bin. Noise was also added to FCS curves, with noise levels determined using noise levels from experimental FCS curves acquired under comparable conditions (Starchev, Ricka et al. 2001). The ACF acquisition time, *T*, for all calculated data sets was 60 seconds.

TCSPC histograms and FCS autocorrelation curves (ACFs) were analyzed using custom and native global analysis routines in Igor Pro. For τFCS analysis, a TCSPC histogram and ACF are considered together as one independent τFCS data set. All fits in Igor Pro were performed using a Levenberg-Marquardt non-linear least squares algorithm which minimizes the χ^2 value, for a τFCS data set defined as:

$$\chi^2=\left(\sum_{i=0}^{k-1}\frac{\left(F_{hist}^{data}\left(x_i\right)-y_i\right)^2}{\sigma_{decay}\left(x_i\right)}+\sum_{i=k}^{k+l-1}\frac{\left(G\left(x_i\right)-y_i\right)^2}{\sigma_{fcs}\left(x_i\right)}\right) \tag{5}$$

where *k* and *l* are the number of data points in the lifetime histogram and the calculated autocorrelation function respectively.

We present two different approaches for data analysis. The first is applicable when a τFCS data set is available for only a single sample condition, e.g. a single concentration ratio, which is a common experimental scenario. In this

case, the molecular concentration and molecular brightness were linked for FCS and fluorescence lifetime data. Stable fitting required independent measurement of the fluorescence lifetime, molecular brightness, and diffusion coefficient for one sample component – and those values were fixed during curve fitting. A second analysis approach is also used, when τFCS data sets for all concentration ratios were fit simultaneously with a single set of global parameters linked across *all* τFCS data sets and separate local parameter sets associated with each τFCS data set.

5. RESULTS

The primary goal of this work is to demonstrate how experimental resolution and model discrimination capabilities in FFS can be dramatically enhanced by using global analysis of FLIM and FCS data. We thus show curve fitting results for computed FCS data sets for a mixture of two fluorescent molecules, each of which has the same diffusion coefficient and the same molecular brightness. A standard FCS experiment would not be capable of identifying the presence of the two sample components, nor of accurately recovering their concentrations and other physical properties. The fluorescence lifetimes of these two molecules are different, with values typical of common fluorescent molecules (τ_1 = 3.92 ns and τ_2 = 1.63 ns). Fluorescence lifetime measurements can easily resolve these two lifetime values but cannot determine molecular concentrations, diffusion coefficients, or molecular brightness values.

We begin with the analysis of individual τFCS data sets for each concentration ratio of the two fluorescent molecules. For the data shown, the concentration of species 1 (SP1) ranges from 50 to 100 nM, and species 2 (SP2) concentration ranges from 50 to 0.2 nM. This provides concentration ratios, reported as C_1/C_2, spanning across almost three orders of magnitude. Fitting of τFCS for a single sample condition requires *a priori* knowledge of the fitting parameters for one of the two sample components. Here we assume that the lifetime, molecular brightness, and diffusion coefficient of the SP1 molecules (τ_1, ψ_1 & D_1) can be measured independently thus serve as fixed fitting parameters during the τFCS analysis. These parameter values are shown in Fig. 2.

The τFCS fitting results are shown in Fig. 2 (squares) as are the known concentrations used in calculations, shown as solid lines. As can be seen in the figure, τFCS analysis accurately returns the molecular concentration, molecular brightness, and diffusion coefficient for each species in the mixtures over a fairly wide range of concentration ratios. Above the concentration ratio of approximately 60 the recovered concentration values becomes unstable in the curve fitting routines and are sensitive to initial parameter guesses due to the covariance of the molecular brightness and concentration parameters. These instabilities can also been seen in the recovery of the diffusion coefficient (Fig. 2D) and brightness (Fig. 2C) of SP2, which are otherwise quite accurate at lower concentration ratios. TCSPC measurements can accurately determine the fluorescence lifetimes of multiple sample components independently of

the FCS analysis, so the measured lifetime values do not exhibit similar instabilities except at the highest concentration ratios (Fig. 2B).

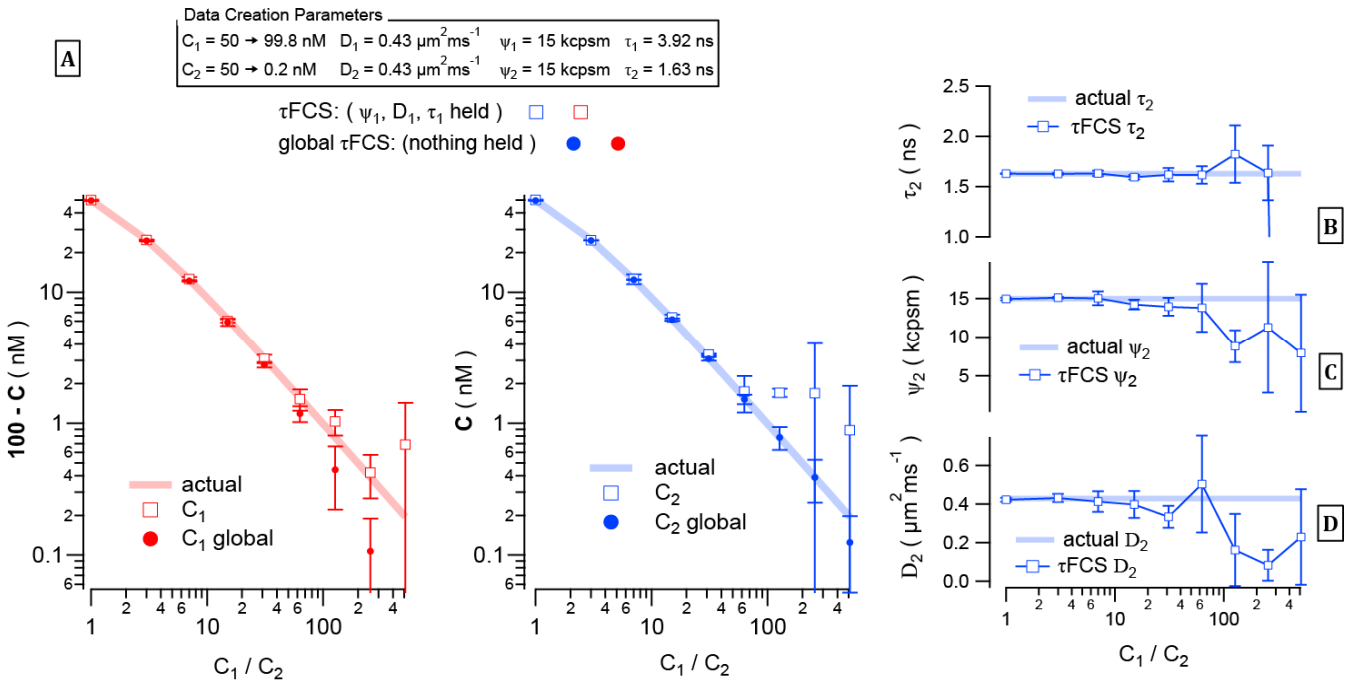

Figure 2 – Determining the accuracy of τFCS by comparison of returned data fitting parameters to values used in data calculations. Hypothetical fluorescent molecules 1 (red) and 2 (blue) are sequentially diluted starting from an equal mixture of 50 nM each (note, species 1 y-axis is plotted as 100 – C to utilize the log scaling). The returned parameter values using τFCS (squares) are accurate and stable up to a ratio of approximately 60, at which point they become unstable and dependent of initial conditions. Returned parameters using Global τFCS (spots) provides an extended range of accuracy and stability, in addition to not requiring any held parameters during fitting.

As shown above, combined lifetime and FCS data for fitting as a single τFCS data set can result in greatly improved resolution of the molecular composition of a sample. The major limitations of the method, as introduced so far, are that *a priori* knowledge of some sample parameters is required for stable curve fitting and the accuracy falls off at the higher concentration ratios. These limitations can be dramatically overcome using the full power of global analysis and curve fitting the entire measurement series with common experimental parameters linked across all of the data sets. The globally linked fitting parameters include the lifetime, molecular brightness, and diffusion coefficient (τ, ψ & D) of each species in the sample. Two additional "local" fitting parameters, C_1 and C_2, are associated with each individual data set to account for the different molecular concentrations of each dye for each concentration ratio.

Parameter	Returned Value	Actual Value
τ_1	3.919 ± 0.006 ns	3.92 ns
ψ_1	14.95 ± 0.03 kcpsm	15 kcpsm
D_1	0.4268 ± 0.0014 $\mu m^2 ms^{-1}$	0.43 $\mu m^2 ms^{-1}$
τ_2	1.626 ± 0.005 ns	1.63 ns
ψ_2	15.07 ± 0.09 kcpsm	15 kcpsm
D_2	0.4293 ± 0.0102 $\mu m^2 ms^{-1}$	0.43 $\mu m^2 ms^{-1}$

Table 1 – Recovered global parameter values using global τFCS to analyze all 9 data sets created to simulate a concentration titration. All values are correct within error, calculated from three repeated calculated titrations and corresponding analyses.

Full global data analysis achieves remarkable accuracy for the concentration of each species across nearly the entire concentration range (Fig. 2A; circles). In addition, the recovered global parameters are exceptionally accurate (Table 1). The sensitivity of these results goes well beyond what is typically achievable in FCS measurements even for cases where the two species diffusion coefficients are sufficiently different to resolve them with FCS alone, and Fig. 2 shows resolution of the concentration of SP2 even when it makes up only a few percent of the molecules in the sample. Moreover, these fitting results were obtained without any constraints on fitting parameters, and the full global analysis approach completely eliminates the need for any molecule specific calibration measurements. None-the-less, the fits returned stable fitting parameters with high accuracy across the entire data set. This offers a tremendous advantage for applications of this method. For complex experimental systems it may be impossible to isolate an individual molecular species for calibration purpose, or calibrations performed under one sample condition (e.g. diffusion coefficients for isolated molecules) may not accurately reflect actual values in a different sample condition (e.g. diffusion coefficients within a living cell).

6. DISCUSSION

We have introduced a new fluorescence fluctuation analysis technique, τFCS, which incorporates micro- and macro-time fluorescence fluctuations observable using pulsed laser excitation and TCSPC hardware. We demonstrated that τFCS can successfully recover concentrations, molecular brightnesses, diffusion coefficients and fluorescence lifetimes from molecular species of identical molecular weight using calculated data sets and simulated noise. These are remarkable results that would not be possible using FCS alone. The ability to accurately recover concentration values from <1% of the overall population goes well beyond previously reported resolution. Moreover, using global-τFCS removes the need to hold any of the fitting parameters fixed during the analysis. The freedom from parameter assumptions and calibrations, together with the marked improvement in accuracy highlight the benefits of global-τFCS.

7. ACKNOWLEDGMENTS

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