Fluorescence correlation spectroscopy using quantum dots: advances, challenges and opportunities

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Semiconductor nanocrystals (quantum dots) have been increasingly employed in measuring the dynamic behavior of biomacromolecules using fluorescence correlation spectroscopy. This poses a challenge, because quantum dots display their own dynamic behavior in the form of intermittent photoluminescence, also known as blinking. In this review, the manifestation of blinking in correlation spectroscopy will be explored, preceded by an examination of quantum dot blinking in general.

1. Introduction

Semiconductor nanocrystals (quantum dots) are on the verge of revolutionizing the field of biophotonics. The extreme brightness and photostability of quantum dots have made single biomolecule imaging almost trivial.1,2 The term “quantum dot”, was coined to represent the reduced dimensionality of nanoscopic crystals made up of 100s to 1000s of unit cells, with core diameters ranging from 1.5 nm–6 nm. At this length scale, the confinement energy of the exciton (electron/hole pair) is stronger than the Coulombic interaction, resulting in quantization of the electronic energy. For optical transitions, excitons are generated through the absorption of light. An extensive and informative review of the optical properties of single quantum dots is given in ref. 3. The optical gap of a quantum dot is size-tunable in the visible to near infrared region of the electromagnetic spectrum. After excitation, emission of a photon occurs in the absence of other relaxation pathways. Conveniently, the dots have broadband absorption, making simultaneous excitation of a selection of different color dots using a single wavelength light source possible. Moreover, they have narrower emission profiles than organic dye molecules, facilitating easier spectral separation of the fluorescence signal. Thus, quantum dots have great potential for multiplexing in biological assay applications, as well as in imaging.

Quantum dots have been successfully used in long-term biological imaging with little or no cytotoxic effects.4,5 To date, quantum dots have been used for optical coding of mammalian cells,6,7 in the formation of biosensors as diagnostic tools for cancer8,9 and in the development of rapid screening techniques using multiplexed hybridization detection of DNA sequences.10 They have been used as donors for fluorescence resonance energy transfer (FRET)11 and as fluorophores in fluorescence lifetime imaging (FLIM)12 because of their long lifetimes (10s of ns) compared to organic fluorophores (1–5 ns). Near infrared dots have been used to map sentinel lymph nodes,14 and dots in the visible region have tracked the diffusion of individual glycine receptors at various synaptic locations15 and so on.16

The chemical research of quantum dots has involved developing luminescent core materials and then adding a higher bandgap shell to the core surface. The resultant core/shell quantum dots were found to have superior optical properties. One of the most widely studied core/shell quantum dots is composed of CdSe/ZnS. CdSe/ZnS quantum dots are made in hydrophobic highly coordinating organic solvents such as trioctyl phosphine oxide (TOPO)17 and in order to render them biologically compatible they need further surface modifications for water solubility and target specificity.18 A schematic diagram of a core/shell quantum dot is displayed in Fig. 1. The ability to solubilize CdSe/ZnS dots in aqueous media by use of an amphiphilic polymer or lipid coating while maintaining a high quantum yield19 has led to their commercialization [Quantum Dot Corp and Evident Technologies]. A peptide coating20 can also render the dots water-soluble, but this formulation is not presently available commercially. Once water solubilized, dots can be further functionalized to target specific cellular surfaces or sites using receptor–ligand interactions.21

Nowhere have the narrow spectral features of quantum dots shown more potential for applications than in fluorescence
(cross)correlation spectroscopy (F(X)CS) of biological systems. FCS and XCS are spectroscopic techniques that rely on photostable fluorophores with high quantum yields to study movement and molecular interactions at the single molecule level. If multiple fluorophores can be excited simultaneously with a single wavelength and their emission is spectrally distinguishable, then the technique is made even more versatile. Quantum dots appear at first to be ideal fluorophores for FCS and XCS applications, but despite their distinct advantages over typical organic dye molecules, their use in FCS has yet to be fully realized. Intermittent photoluminescence or “blinking” of quantum dots has limited their use as an analytical tool pending a physical/mathematical model for the FCS/XCS data, or at least adequate manipulation of their photophysical properties to remove the intermittency of photoluminescence.

Nevertheless, there have been several recent applications of quantum dots in correlation spectroscopy applications. Initially, FCS was used almost exclusively to characterize the concentration and size of biofunctionalized quantum dots. Quantum dots have also been employed in XCS studies of ligand–receptor binding. In both XCS studies, the ligand–receptor investigated was the streptavidin–biotin model system. Hwang and Wohland presented evidence for binding between biotinylated fluorescein and a streptavidin functionalized quantum dot (655 nm). In the other XCS work, two-photon excitation of red and green quantum dots was employed to examine the effect of large nanocrystals on the two-photon excitation of red and green quantum dots functionalized quantum dot (655 nm). In the other XCS work, two-photon excitation of red and green quantum dots was employed to examine the effect of large nanocrystals on the two-photon excitation of red and green quantum dots. Quantum dots have also been employed in XCS studies of ligand–receptor binding. In both XCS studies, the ligand–receptor investigated was the streptavidin–biotin model system. Hwang and Wohland presented evidence for binding between biotinylated fluorescein and a streptavidin functionalized quantum dot (655 nm). In the other XCS work, two-photon excitation of red and green quantum dots was employed to examine the effect of large nanocrystals on the two-photon excitation of red and green quantum dots.

2. Blinking and other photophysical properties of quantum dot photoluminescence

Before examining fluorescence correlation spectroscopy more formally, some background essential to better understand blinking will be introduced. Blinking is observed for many types of single emitters and is a fundamental photophysical property of quantum dots. For quantum dots, the “off” periods in the fluorescence signal occur, limiting long-term signal monitoring, despite continuous excitation conditions. Blinking has been observed on time scales ranging from approximately 200 μs to 100s of seconds and is thought to arise from quantum dot ionization, which occurs even under ambient lighting conditions. Organic dye molecules also have intermittency events which typically follow exponential statistics indicating a single dark, trap state, (often triplet) which leads to well-defined blinking kinetic rates. In contrast, power law distributions are observed for the time-dependent probabilities of quantum dot blinking events, meaning that the events occur over a vast range of time scales. This suggests that blinking is a stochastic process. When averaged over many excitation cycles, blinking will lower the steady state photoluminescence quantum yield. The photoluminescence quantum yield has been shown to be sensitive to fluctuations in the dot’s local environment, suggesting that molecules adsorbed to the dot’s surface may change de-excitation pathways. Changes in the quantum yield have been observed for quantum dot ensembles, either free in solution or immobilized on a surface. Ensemble measurements include reversible photoluminescence enhancement, reversible photobleaching, photo-darkening, electrochromic properties and sub-populations of dark dots. Single dot measurements tend to be related to dynamic changes in photoluminescence and include; blinking, random fluctuations of the emission wavelength and intensity-termed spectral diffusion, fluctuating quantum yields and long-term spectral shifts correlated with permanent photo-bleaching.

The photoluminescence intensity (count rate) per dot, an example of which is displayed in Fig. 2a, can show sensitivity to the mode of excitation (pulsed or continuous, one or two photon), the intensity and energy of the excitation source (on resonance or above-band excitation), as well as the exposure time and the presence of strong oxidizing or reducing agents. A number of non-radiative relaxation pathways including ionization and ionization suppression mechanisms exist in quantum dots affecting their observed photoluminescence properties and excited state lifetimes. These pathways may be photo-, thermal- or chemically induced and either prevent or promote radiative recombination of the exciton and diminish or enhance the quantum yield making energy transfer mechanisms considerably more complex in nanocrystals than either molecular systems or bulk materials. Correlation of these other observables with blinking is still under investigation; “off” time distributions appear to be insensitive to environmental conditions however “on” times deviate from power law statistics and even switches to exponential statistics in the absence of the ZnS capping agent. The power law distribution of “off” times (Fig. 2b) suggests a random renewal process for the recovery of the quantum dot back into the bright state. In fact, one of the most surprising features about quantum dot blinking is that in an ensemble of dots every dot is statistically equivalent which is unanticipated given the ionization mechanism proposed, as this should show obvious environmental sensitivity. Systematic studies into the effects of environment and excitation rates on ensemble fluorescence intermittency using fluorescence correlation spectroscopy will prove useful.

3. FCS background

One method to analyze the fluctuations in fluorescence intensity resulting from quantum dot blinking is to calculate the
temporal intensity autocorrelation function.\textsuperscript{47} In the case where the quantum dots are immobilized \textit{via} coupling to a surface, the resultant autocorrelation function should represent time correlations solely resulting from photophysical changes, whereas for mobile dots diffusion will also affect the autocorrelation decay. In general, the autocorrelation analysis of fluctuations in the fluorescence intensity is also known as Fluorescence Correlation Spectroscopy (FCS).

FCS is a sensitive technique employed mostly for the detection of freely diffusing species. In the case of mobile emitters, the fluorescence intensity time trace, or histogram, of a well-defined interrogation volume will show intensity fluctuations due to the exchange of fluorophores.\textsuperscript{47} Transit times are a function of both solution temperature and viscosity as well as the hydrodynamic radii of the emitters.\textsuperscript{47} If only a few fluorophores are in the excitation volume at any one time, then fluctuations due to diffusion are statistically significant\textsuperscript{47} and any changes in the emission characteristics of the fluorophore give rise to additional fluctuations, which will affect the FCS signal. Information such as local concentrations, mobility coefficients and rate constants for inter- or intra-molecular reactions can be extracted by analyzing the fluorescence autocorrelation decay.\textsuperscript{47}

As mentioned above, periodic fluorescence intensity fluctuations may be detected using auto-correlation analysis. This analysis is akin to integrating the product of the fluctuation in fluorescence intensity from the average value at a time \( t \), \( \delta F(t) \), and that at a time \( (t + \tau) \), \( \delta F(t + \tau) \), where \( \tau \) is a variable lag time. The product is averaged over \( t \), to give the auto-correlation value, \( G(\tau) \) as a function of \( \tau \). In order to compare the auto-correlation functions from different times or samples, they are normalized using the average intensity, \( \langle F \rangle \). The normalized ensemble averaged fluorescence intensity auto-correlation function therefore is

\[
G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} = \frac{\langle F(t)^2 \rangle - \langle F(t) \rangle^2}{\langle F(t) \rangle^2} - 1
\]

(1)

Rather than measuring \( \delta F(t) \), one often measures the changes in fluorescence intensity, \( F(t) \). An autocorrelation of this parameter delivers \( G(\tau) + 1 \), which can be plotted against \( \tau \) to yield the autocorrelation decay. For \( \tau = 0 \), the autocorrelation amplitude can be defined as:

\[
G(0) = \frac{\langle F(t)^2 \rangle - \langle F(t) \rangle^2}{\langle F(t)^2 \rangle} = \frac{\text{variance}}{\langle F(t)^2 \rangle}
\]

(2)

Since both the variance and the mean fluorescence intensity are proportional to \( N_{\text{Ave}} \),

\[
G(0) = \frac{1}{N_{\text{Ave}}}
\]

(3)

where \( N_{\text{ave}} \) is the average number of emitting species in the interrogation volume. When the lag time is plotted logarithmically, the autocorrelation decay consists of three characteristic regions defined by short, long and intermediate values of \( \tau \). A typical autocorrelation decay for a non-blinking organic

Fig. 2 (a) Typical intensity time trace of immobilized CdSe(ZnS) quantum dot fluorescence intermittency (blinking) at room temperature and (b) at 10 K. (c) The normalized off-time probability distribution for one quantum dot (diamond) and averaged over 39 quantum dots (triangle). Inset shows the distribution of fitting values for the power law exponent in the 39 quantum dots. The straight line in the main graph is a best fit to the average distribution to eqn (5) with exponent, \( \alpha \sim -1.5 \). Reprinted with permission from K. T. Shmizu, R. G. Neuhauser, C. A. Leatherdale, S. A. Empedocles, W. K. Woo and M. G. Bawendi, \textit{Phys. Rev. B}, \textbf{63}, 205316, 2001. Copyright (2001) by the American Physical Society.
dye molecule is given in Fig. 3. For very short lag times diffusion is negligible and \(G(t) = G(0)\) (constant). For very long lag times there is no correlation and the auto-correlation function decays to a constant (either 0 or 1 depending on whether \(G(t)\) or \(G(t) + 1\) is plotted). On the millisecond time scale and in the absence of photochemical processes, diffusion through the focal volume (~1 \(\mu\)m\(^3\) for confocal applications) occurs dominating the auto-correlation function, which decays rapidly. Changes in the shape, amplitude or characteristic decay time of the auto-correlation function may provide information on underlying chemical, photophysical, conformational or other changes for the fluorophore under investigation.\(^47\) In FCS, the concentration of emitters is kept low so that movement through the focal volume may be easily detected. To improve statistical accuracy, the auto-correlation decay is often built from hundreds of thousands of events.

The rapid growth in applications of FCS occurred after FCS was applied using confocal microscopy. Confocal microscopy allows for a very small volume of solution to be interrogated (~1 fl). Small interrogation volumes are needed such that only 1–100 molecules are examined. This small number results in detectable fluctuations, which are easily analyzed using FCS. A typical TPE-FCS experimental set-up is shown in Fig. 4. The light source is a pulsed femtosecond laser, which allows for the high peak intensities needed to drive two-photon absorption. The laser beam is expanded to overfill the back aperture of a high numerical aperture (NA > 0.9) objective lens. This results in a diffraction-limited focal spot. TPE confines the excitation volume, the fluorescence from which is collected back through the objective lens and directed to Si avalanche photodiode detectors. These detectors have the high quantum efficiency (>60%) and low background dark noise necessary for single molecule detection. The count rate signal from the detectors (single photon counting mode) is usually directed to a hardware correlator card (ALV Langen or Correlator.com), which calculates the autocorrelation and cross-correlation signals. Event counters can also be used to collect the count rate histograms from which the correlation decays can be calculated, off line. The only difference between this set-up and the one for single photon excitation is that the laser used is operated in continuous mode and a pinhole is placed at the image plane in front of the detector in order to define the interrogation volume.

4. Details of previous FCS studies on quantum dots

Only a handful of fluorescence correlation studies on quantum dots have been published to date\(^22\)–\(^25\),\(^48\),\(^49\) despite the potential for insight into the blinking mechanism. For example, since the \(1/G(0)\) represents the number of emitters in the interrogation volume, a comparison of \(G(0)\)’s versus laser power would provide insight into light-induced changes in quantum dots. The most straightforward change would be in the number of dots excited versus light intensity. Doose et al.\(^22\) compared the photophysical and colloidal properties of different biocompatible quantum dots using FCS. In their study, \(G(0)\) values were examined at different laser powers for several different types of water-soluble quantum dots and compared these results with those from fluorescent beads and the organic dye rhodamine-6-green (R6G). The latter of which is a common fluorescence reference standard. Using a 532 nm continuous wave (cw) laser to interrogate a 1 femto-litre focal volume in the power range 1–1000 \(\mu\)W, they found that as a function of laser power, \(G(0)\) decreases substantially more for the dots than it does for the beads or R6G. The accepted rationalization for a decreasing \(G(0)\) as a function of increasing laser intensity is an increase in the interrogation volume resulting from excitation saturation effects.\(^50\)–\(^53\) Using alternating laser excitation scheme fluorescence correlation spectroscopy (ALEX-FCS), Doose et al.\(^22\) were able to rule out optical trapping, but not power-dependent changes in blinking, as a source for the large decrease in \(G(0)\) with excitation power. Additionally, they observed abnormally large fluctuations in \(G(0)\) over a series of measurements at low laser power. This could indicate heterogeneity in particle brightness and was attributed to the presence of small aggregates.\(^22\) Although blinking may be responsible for these observations, it is difficult to prove, because the autocorrelation function for both blinking and diffusion has not been parameterized. Moreover, it has been suggested that the autocorrelation decay including quantum dot blinking may not be indistinguishable from one for diffusion only.\(^54\) However, observations of the changes in the shape
of the autocorrelation decay, particularly at short lag times, for quantum dots at higher excitation rates, suggests otherwise. An example of the change in autocorrelation decay shape found by Weiss and coworkers is reproduced in Fig. 5. They employed Monte Carlo calculations of diffusing and blinking dots to simulate the anomalous shape of the auto-correlation function. Unfortunately, no unique set of blinking parameters for a given data set could be found.

Larson et al. observed similar results for the changes in $G(0)$ values versus excitation rate (i.e. laser power) using two-photon excitation FCS and attributed this to excitation saturation. The auto-correlation curve for the quantum dots in their study also changes shape with increasing laser power (Fig. 1b. Larson et al.), indicating that some photo-induced process is occurring. Saturation can account for anomalous changes in the value of $G(0)$ versus power, but not for changes in the shape of the autocorrelation decay. Such anomalous forms of the autocorrelation decay in the 1 μs–1 ms lag time range, (~1 FL interrogation volumes) can only result from concomitant periodic fluctuations in the fluorescence intensity.

Given the above indirect observations of anomalies in the autocorrelation decays, a direct observation of blinking in mobile dots was needed. Confocal fluorescence coincidence analysis (CFCA) measurements were applied to verify that quantum dots blink when mobile in solution. The approach premise used by Yao et al. was to label dots with organic dyes in order to create an intensity trace for the dots transitioning the interrogation volume in one detection channel and then observe quantum dot luminescence in the other. Since the organic dye labels will not blink under low excitation rates, events in the count rate histogram for the dye will reflect the total transit time for the dot. Streptavidin coated 525 nm dots were labeled with biotinylated Alexa fluor 594 dye molecules. Both dots and dye labels were excited using a 488 nm laser (100 μW, cw) and were observed as bursts in the photon count rate histogram for either the red channel (labelled dark dots), the green channel (unlabelled bright dots) or both channels (labelled bright dots). Intermittency in the green channel due to quantum dot blinking was observed and the probability of observing a blinking event is increased from 14% to 35% by lengthening the transit time from 1 ms to 10 ms, via increasing solution viscosity. Sub-populations of dark dots were also measured using two-photon excitation correlation spectroscopy and agreed with the results from CFCA. The relative numbers of dark and bright dots remained unchanged with respect to transit time, suggesting that dark dots are not in a temporary “off” state. The overall percentage of bright dots in a sample ranges from 30% to greater than 90%, depending on the quantum dot production batch, and follows the trend with the bulk quantum yield of the dots used in this study.

In contrast to the above experiments, the majority of quantum dot blinking data has been recorded for immobilized dots. Blinking behavior is often used as the distinguishing criteria with which one identifies single emitters. However, the observation of anti-bunching in photon correlation measurements is now considered a more definitive indicator that a single quantum emitter is being investigated. Messin et al. reported that the correlation curve of a single CdSe (i.e. no shell) quantum dot intensity time trace reflected antibunching at short lag times and was relatively horizontal.
between 200 ns and 100 μs indicating the absence of blinking in this time range. Beyond 100 μs the curve decayed slowly until the measurement time was approached and the decay fell abruptly, but not asymptotically to zero. This suggests that blinking of mobile dots under similar circumstances would be challenging to deconvolute from the diffusion influenced autocorrelation decay, as was previously proposed by Pelton et al.\textsuperscript{54}

In general, since quantum dot blinking is a stochastic process, there is no characteristic time scale over which the process occurs and the effect of quantum dot blinking on the ensemble averaged auto-correlation function has not been derived parametrically.\textsuperscript{56} The auto-correlation function measured for any portion of the intensity time trace will be different than the next, exhibiting ergodicity breaking for the single emitter due to the random nature of the intermittency. It will be of interest to determine whether or not the anomalies observed for ensemble average fluorescence autocorrelation decay for mobile quantum dots behave in a similar fashion as the ensemble average autocorrelation decays for immobilized quantum dots.

5. Further examination and modeling of quantum dot blinking

(a) Fluorescence intensity histogram analysis

The time traces of fluorescence intensity (or count rate histograms) for single immobilized quantum dots can be analyzed directly. Such analysis reveals that blinking is best described as a stochastic renewal process, since “on” and “off” time probability density distributions follow power law statistics with exponents between −1.4 and −1.9, depending on the measurement conditions.\textsuperscript{35,44,57,58} The probability density is given as:

\[
P(t_{\text{off/on}}) = \frac{\text{No. of times} “t_{\text{off/on}}”}{\text{Total time} “t_{\text{off/on}}”}
\]

and the power law distribution is

\[
P(t) = At^\gamma
\]

where \(A\) is a proportionality factor. The smallest “on” or “off” time that may be observed is limited by the width of the measurement window (data collection time bin size) or time resolution of the experiment. Bin sizes are chosen so that a distinction can be made between a period where a photon has not been emitted and a dark period where photon emission is expected, the latter of which defines a true “off” time and the former represents the emission lifetime. Typically bin sizes range from 50 μs to 10 ms depending on the count rate of the sample which varies considerably with excitation conditions. While blinking “off” occurs more frequently with increasing temperature and laser intensity, recovery from the “off” state appears to be insensitive to experimental conditions.\textsuperscript{35,44,59} A maximum “off” time is difficult to define or observe from a single count rate histogram as the duration of the measurement is limited by long term drift of the apparatus\textsuperscript{35} as well as photobleaching of the dot.\textsuperscript{43}

Since no upper bound for the “off” times could be determined from the single intensity time traces, the renewal process was considered to be non-ergodic and unpredictable.\textsuperscript{58} Non-ergodic behaviour implies that the auto-correlation analysis of the fluorescence intensity fluctuations of a single immobilized emitter, averaged over a sufficiently long observation time will not be the same as the auto-correlation function of an intensity time trace of an ensemble of emitters sampled for a much shorter time period. In contrast to this prediction, Wiseman and coworkers\textsuperscript{60} using image correlation spectroscopy (ICS), were able to show, in principle, that individual blinking parameters were extractable from the auto-correlation analysis of large ensembles of immobilized dots. They showed that the auto-correlation function of the count rate histogram from single emitters were reproducible, provided histograms with a well represented range of “on” and “off” times were chosen. In a different but similar study, Pelton et al.\textsuperscript{54} used Fourier analysis of the time fluctuations of ensemble measurements. For a limited frequency (or time) range, they could extract blinking statistics of single dots for immobilized and mobile emitters. These latter studies\textsuperscript{54,60} and observations of fluorescence decay for immobilized ensembles\textsuperscript{32} indicate that a maximum “off” time in quantum dot blinking does exist and these systems are not completely non-ergodic.

Recently, a method for determining the maximum values for the “on” and “off” times via recording the fluorescence decay of an ensemble of immobilized quantum dots was published by Bawendi and co-workers.\textsuperscript{32,33} The maximum “off” times determined from their fluorescence decays were on the order of many 1000s of seconds, much longer than typical observation times for single dots measurements. It appears that blinking occurs over ten orders of magnitude in time (10⁻⁶ up to 10⁴ s).

(b) Spectral analysis and blinking

Since most experimental set-ups used to examine the fluorescence of quantum dots employ band pass emission filters, one must examine the possibility that an off event is equivalent to a transient shifting of the emission spectrum beyond the filter’s cut-off wavelength. Blinking has been associated with small reversible spectral shifts of a few nm which is also known as spectral diffusion,\textsuperscript{39,40} in contrast to much larger spectral shifts (10s of nm) which occur prior to permanent photobleaching events.\textsuperscript{43} These small shifts should not significantly affect detectability in a typical fluorescence experiment.

(c) Effect of environment/capping on other photophysical characteristics

Blinking statistics suggest that the rate of sampling into the “off” state is increased with increasing temperature or laser power, suggesting a barrier which activation energy is required to cross.\textsuperscript{35} Recovery from the “off” state appears to be independent of all environmental influences. Systematic investigations into the variance of the power law exponent with environment are required. A value other than −0.5 for the power law exponent of the “on” and “off” time distributions infers that the process is not purely random, and that other secondary interactions are occurring.\textsuperscript{61} The rate of photodestruction of a quantum dot can be directly correlated to the
number of layers of capping material limiting the “off” times present, being much slower for increasing numbers of layers.\textsuperscript{42} This suggests that capping layers are not grown epitaxially but rather they contain grain boundaries through which oxygen may diffuse into the core in order to promote destructive oxidation.\textsuperscript{42}

Other phenomenon observed for quantum dots making them different from organic dye molecules include reversible photo-induced photoluminescence enhancement,\textsuperscript{29,30} reversible photobleaching and photo darkening,\textsuperscript{31,32} as well as fluctuating quantum yields\textsuperscript{23,28} and fluctuating and multiple excited state lifetimes.\textsuperscript{31,62–65} Zhang \textit{et al.}\textsuperscript{66} using intensity changepoint reconstruction of the single emitter count rate histogram could determine a positive, but not linear, correlation of intensity with lifetime. Their results also indicate that a pure stochastic model for quantum dot fluorescence intermittency is inadequate and that a distribution of emission states must exist. This is similar to the conclusions of Watkins and Yang\textsuperscript{67} and Margolin \textit{et al.}\textsuperscript{58} that the underlying mechanism for blinking in quantum dots is the result of a distribution of trap sites in either energy or space.

6. **Excitation rate dependence of anomalous fluorescence autocorrelation signals**

In our group, we have examined the two-photon excitation fluorescence autocorrelation decays of mobile quantum dots. We observe an anomalous shape in the autocorrelation decay, which appears to be a function of the excitation rate. Typical laser power dependent autocorrelation decays are displayed in Fig. 6. This figure shows a series of auto-correlation decays which depict the dependence of green quantum dot autocorrelation decay on laser power \textit{(i.e. excitation rate)}. As the laser power is increased, a change appears in the autocorrelation function in the 0.01 to 0.5 ms region. For the green quantum dots, this effect then reaches an asymptote at approximately 60 mW.

In Fig. 7, two autocorrelation decays are displayed for closer inspection. These are from a 2 nM solution of streptavidin-functionalized green quantum dots ($\lambda_{em} = 525$ nm) in borate buffer, at two different laser powers of 10 mW and 70 mW, and are plotted on two different amplitude scales. This is necessary since the correlation function amplitude, $G(0)$, is strongly dependant upon laser power, and differences in the shapes of the decay curves at short lag times, ($\tau$), are more easily compared in such a plot. The 10 mW curve is shown in black squares and is easily fitted using the normal expression for diffusion.\textsuperscript{68}

$$G(\tau) = G(0) \left(1 + \frac{8D\tau}{\omega_R^2} \right)^{-1/2} \left(1 + \frac{8D\tau}{\sigma_A^2} \right)^{-1/2}$$ (6)

where, $\tau$ is the lag time, $D$ is the diffusion constant, $\omega_R$ is the laser beam waist at its focus and $\omega_A$ is the depth of focus. The concentration and diffusion coefficient of the 525 nm
be given in general:

$$G(t) = G_{diff} (t) + G_{blink} (t)$$

where $F$ represents the fraction of dots with detectable fluctuations not described by the diffusion (i.e., eqn (6)). $G_{blink} (t)$ is the function that models the anomalous shape of the autocorrelation decay. The form of $G_{blink} (t)$ that best describes this shape was found empirically to be:

$$g_{\text{blink}}(t) = A t^{-2}$$

To fit the model function to the data, parameters $\alpha$, $A$, $F$, $\omega_R$ and $\omega_A$ were allowed to float. The parameters were optimized via least squares analysis using the software package, Origin.

The resulting fit using eqn (7), is given for the 70 mW data, as black line in Fig. 7.

Significantly, the value of $\alpha = 1.5$ was found for all anomalous autocorrelation decay fits. Although this region of the autocorrelation decay is where triplet state blinking effects appear for organic molecules, the short time behavior for the quantum dots cannot be modeled adequately using a standard singlet–triplet type coupling, which results in an exponentially decaying contribution to the autocorrelation function.69

At short times, changes in the value of $G(t)$ are dominated by blinking ($t^{-1/2}$), because the diffusional contribution to $G(t)$ is constant. At times approaching the transit time, the decay of $G(t)$ is dominated by diffusional decay, which goes like $t^{-3/2}$. The change in excitation volume due to the higher laser intensity is accounted for by also allowing the parameters $\omega_R$ and $\omega_A$ to float, while holding the concentration and diffusion coefficient constant at those values obtained with eqn (6) at 10 mW.

The previous study of mobile quantum dots by Doose et al.22 examined the possibility of factors other than blinking, such as optical trapping and changes in concentration that could influence the shape of the autocorrelation decay in a laser power dependent manner. They determined that the effect on the autocorrelation decay could not be explained without accounting for blinking. To further test whether or not the changes in the autocorrelation decays observed in the present study result from blinking, fluorescence cross-correlation spectroscopy of physically bound, but spectrally distinct pairs of dots was used. Blinks should not be evident in the cross-correlation signal unless the blinking between two joined dots is synchronized.

The cross-correlation decay for physically bound dots of different colors was measured for a series of laser powers. The resulting cross-correlation decay curves for a solution of 605 nm biotinylated dots (QD605) and 525 nm streptavidin coated dots (QD525), as a function of laser power, is given in Fig. 8. The cross-correlation amplitude of two linked dots does
not exhibit the “blinking effect” even with increased laser power. This is not surprising since the blinking events from each dot in the pair are not expected to be synchronized. The QD_R QDs pair displayed no other evidence of electronic coupling (i.e. no FRET signal between them). The cross correlation data were fitted using eqn (6), which is a reasonable approximation, assuming mostly 1 : 1 association of the red and green dots. Eqn (6) does not explicitly account for saturation effects, but appears to model the cross-correlation data adequately by allowing the volume parameters to float. The four cross-correlation plots are, in fact, from the same solution and laser powers as those presented in Fig. 6. The behavior in the absolute amplitude of the correlation functions is similar. In going from 9 mW to 203 mW, the $G(\tau)$ at $10^{-5}$ ms drop by a factor of slightly greater than 3. Such behavior is rationalized in terms of an increase in the TPE observation volume due to saturation of two-photon excitation. Therefore, it is clear that the dots in the power studies are under the influence of excitation saturation. However, there is no evidence that saturation will lead to the changes in the shape of the autocorrelation decay signal at short times observed both by us (Fig. 6) and Weiss and co-workers (Fig. 5).

If we consider that the anomalous shape of the autocorrelation decays shown in Fig. 5 and 6 results from quantum dot blinking and not artifact, then it is interesting that the lag time ($\tau^2$) dependence of that part of the autocorrelation decays, with $x = 1.5$ is similar to the dependence observed by Orrit and coworkers. They reported a dependence of $\tau^{-0.3}$ for single uncapped, immobile CdS quantum dots. We measure the ensemble average behavior, rather than the autocorrelation decay for a single dot. Autocorrelation analysis of ensembles of CdSe quantum dots on surfaces has revealed similar behavior. However, a different empirical model was used for evaluation of lag times, which were recorded for the range 0.1–30 s. As noted above, Weiss and co-workers observed similar anomalous autocorrelation decay shapes for mobile CdSe dots using one photon excitation FCS. Moreover, they observed that the anomalous shape depended on excitation rate (i.e. laser power applied to the sample).

It would be of interest also to determine whether or not the changes in short time correlation persist at longer times. However, changes due to blinking are difficult to deconvolute from the diffusional decay of the correlation functions. The longer time correlation function behavior can be examined by comparing the change in TPE volume dwell times, $\tau_D$, for autocorrelation and cross-correlation decays as a function of excitation rate. The dwell time in the excitation volume is defined as:

$$\tau_D = \frac{\omega_r^2}{8D}$$

(10)

Given that the cross-correlation signal is independent of blinking dynamics, the dependence of dwell time versus laser power should reflect only excitation saturation. In the case of the autocorrelation decay, blinking dynamics with the same characteristic time as the diffusional exchange in the excitation volume could be convoluted and therefore, are potentially measurable. For these measurements, we have again used the same solution as that used to collect the data displayed in Fig. 6 and 8. In this solution a low degree of binding was maintained such that the autocorrelation decays represent mostly free red and green quantum dots. Thus, the absolute values of the dwell times are different for the red dots, green dots (autocorrelation) and red–green bound dots (cross-correlation). For comparison of $\tau_D$ versus excitation rate, the dwell times were normalized to their values at the lowest laser power, 9 mW. In Fig. 9 (tau-power), we find that the normalized dwell times all follow the same power dependence within measurement error. Thus, it appears that the phenomenon that results in an anomalous shape of the autocorrelation signal at short times, does not significantly affect the autocorrelation signal at long lag times.

The similarity of excitation rate dependence between our results and those of Weiss and coworkers is striking, despite the different methods of excitation: two-photon versus one-photon, respectively. Additionally, for an ensemble of surface-bound quantum dots, Wiseman and workers also observed a dependence of the blinking derived autocorrelation function on excitation rate. These FCS-based results taken together with fluorescence histogram analysis strongly support that blinking behavior is related to excitation rate. For mobile quantum dots, the interrogation window is limited by the average transit time through the FCS detection volume, typically a few milliseconds. The dots’ behavior over this time window is then averaged over the many transit events of different dots. The main advantage of FCS analysis of quantum dots is that rare events in the time regime of 1 μs to 1 ms can be accumulated and therefore detected. This is challenging for a typical fluorescence histogram collection.
experiment, where blinking probabilities are reported over $10^{-3} - 10^2$ s.\textsuperscript{35,44,57,58}

7. Closing remarks

There are several intriguing possibilities for FCS to add to the understanding and mitigation of quantum dot blinking. More autocorrelation analysis in the time range 1 μs–1 ms of surface-bound ensemble quantum dots would be illuminating. The one study thus far\textsuperscript{38} reported mildly decaying autocorrelation data in this time range for uncapped CdS quantum dots. The addition of surface active agents to the solution should affect blinking behavior as measured by FCS. It will be interesting to determine whether or not there is a difference between reducing and oxidizing quantum dot surfaces. www.nsti.org/publ/MSM2002/382.pdf.

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References


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