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Environment-Dependent Single-Molecule Spectroscopy

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Abstract
(Single-Molecule Spectroscopy, Fluorescence Intermittency, Pearson’s Correlation Coefficient Analysis)

The dependence of frame rate on fluorescence correlation studies of “on” events in CdSe nanoparticles is investigated. Average Pearson’s correlation coefficient (r) values decrease at a greater rate for particles imaged at a frame rate below 70 s⁻¹ than at frame rates between 70 s⁻¹ and 200 s⁻¹. To better understand this trend, the same particle is imaged for all frame rates and data shows high variability in r values. Overall, data suggests that (1) application of correlation analysis on future studies pertaining to the influence of environment on nanoparticle fluorescence will need to be done on the same particle and (2) r values may only be comparable for results obtained at the same frame rate for the same particle. The data suggests that r values themselves may not be useful to analyze correlations between on events but a change in the r value for the same particle imaged at a certain frame rate under different environmental conditions may give some insight into environmental influences on fluorescence.
ENVIRONMENT-DEPENDENT SINGLE-MOLECULE SPECTROSCOPY

By

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I. Background

a. Single-Molecule Spectroscopy (SMS)

Single-molecule spectroscopy is the observation of only one molecule in a typically very dilute sample using tunable optical radiation. The sample is irradiated by a light source, usually a laser, and the absorption or fluorescence is measured by a detector, such as a charge-coupled device (CCD). Standard spectroscopic techniques yield ensemble-based measurements where the information received by the detector is the average measurement of a parameter of interest from a large number of molecules in a sample. Since all the molecules in a sample are not usually identical to each other, a lot of information is lost in the ensemble-based measurement. Single-molecule spectroscopy eliminates this by providing access to a wide distribution of populations including low-probability events. The shape of this distribution can provide additional information especially when the sample is heterogeneous, as is common in biological samples (i.e. protein sample containing different folded states, etc). Using a home-built SMS microscope in the Knappenberger group, data can be acquired as images and/or as fluorescence trajectories for single CdSe quantum dots, Figure 1. A more detailed discussion of the current analysis of such a trajectory can be found in the “Fluorescence Intermittency” section.
SMS measurements are achieved by ensuring that only one molecule is irradiated in the volume probed by the laser. This is usually achieved by diluting the sample to very low concentrations, roughly $10^{-10}$ mole/liter in a probed volume of $10\mu m^3$. Additionally, good SMS measurements must provide a signal-to-noise ratio greater than one for a single molecule. Using fluorescence-detected SMS, the Knappenberger group has been able to routinely achieve a signal-to-noise ratio (SNR) that exceeds 7 for semiconducting nanostructure samples. For nonlinear SMS data acquired using a laboratory-built second harmonic generation (SHG) microscope, SNR exceeds 5. Such high SNR’s is significant because good data at the single particle level can be acquired, opening up many experimental possibilities from physics to biological systems.

b. Semiconducting Nanocrystals

Since this study uses fluorescent quantum dots to develop SMS experimental and data analysis methods, a brief discussion on what is currently known will follow. Semiconducting nanocrystals are small particles in the range of 1-100nm that possess optical properties that are dependent on the size of the particle. The dependence of
particle size and shape on the observed optical properties of these structures is explained by band gap energy ($E_g$) theory.

The $E_g$ is the minimum amount of energy required to promote an electron in bulk semiconductors from the valence band of the system to the conduction band (Figure 2). The energy gap will vary depending on the material used, and a list of $E_g$ values for commonly used materials is shown in Table 1. If the $E_g$ is in the range of 1-3 eV, the energy to promote the electron can be supplied by a photon of visible light as is the case for various sizes of CdSe quantum dots. Once this electron is excited to the conduction band, it leaves behind a positively charged orbital hole in the valence band. This electron-hole pair, known as the exciton, can be mobilized in the presence of an electric field. The electron can also relax back down to the valence band, eliminating the exciton, and emitting a photon of light.

![Figure 2. Schematic of electron-hole pair in semiconductors. The electron can relax back down in one of two ways: (1) via radiative recombination in which the electron relaxes back down by emitting a photon of light or (2) through non-radiative recombination by dissipating its energy through lattice vibrations.](image-url)
The size of the exciton varies from 1 nm to more than 100 nm depending on the material. If the size of the nanoparticle is smaller than the size of the exciton, then the charge carriers become spatially confined raising the energy of the system. This is known as quantum confinement, and it is under these conditions that a nanoparticle shows size-dependent absorption and fluorescence spectra. By varying the size of the nanoparticle without exceeding the exciton diameter, a narrow range of different $E_g$ for a particular material can be achieved providing the ability to tune particles of the same material (with different sizes) that absorb and emit in different parts of the electromagnetic spectrum. A particle that emits in the visible region of the spectrum, as is the case for CdSe quantum dots, can be useful for biological labeling and imaging.

Particle shape is another parameter that can be manipulated to control optical properties. Most nanoparticles are synthesized as colloids. Since colloids are synthesized in solution, they provide a lot of control over size and shape and thus have
been largely used to understand size-dependent optical properties and are the type used in this study. These nanoparticles can be synthesized as three-dimensional structures (termed quantum dots), as two-dimensional structures (nanorods), or as one-dimensional structures (quantum wells). The different dimensionalities confine the exciton to varying degrees. In general, by increasing the number of confined dimensions (quantum dots having the highest degree of dimensionality) a wider range of tunability in the band gap is possible.

The surface properties of nanocrystals are another important aspect of nanocrystal optical properties. The atoms on the surface of the nanocrystal are incompletely bonded within the crystal lattice which leaves an orbital available for trapping charge carriers at the surface. This presents a problem for applications that depend on fluorescence emission because the trapping of charge carriers increases the probability of nonradiative decay events. Passivation, or coating, of the nanocrystal surface with other atoms or molecules, as is done with CdSe/ZnS core/shell quantum dots, can prevent nonradiative decay events and increase their fluorescence emission. Interestingly, the fluorescence emission from single quantum dots is actually intermittent and will be discussed further in the next section.

Semi-conducting nanoparticles are currently under intense investigation for a variety of applications ranging from electronic devices to food science. Huynh et al. have implemented nanorods into solar cell technology for higher working efficiency. By manipulating nanorod length and radius, a photovoltaic device was assembled from solution with an external quantum efficiency of over 54%. Other applications involve the use of semiconducting nanocrystals for biological staining and diagnostics. Due to their tunable emission spectrum and their chemical stability, in many cases they prove to be more useful than conventional fluorophores as biological labels. M. E. Ali et al. have used gold nanoparticles to reliably detect pork adulteration in meatball.
formulation. This colorimetric technique is expected to find applications in food analysis, genetic screening, and homology studies.

c. Fluorescence Intermittency

Fluorescence intermittency, or blinking, is the random interruption in fluorescence emission observed at the single-molecule level when the sample is excited by light. This blinking behavior is not exclusive to quantum dots—other studies have investigated intermittency in single-chromophoric dye molecules and multiple-chromophoric conjugated polymer molecules. For CdSe quantum dots, this behavior is reflected in the fluorescence trajectory acquired for a single dot, shown in Figure 3a, where the fluorescence intensity fluctuates over time. This data is analyzed by taking the fluorescence trajectory and converting it into a binary data set by assigning a threshold three times the standard deviation of the average of the background counts. This divides the data into two broad categories (Figure 3b). For intensity count levels above this threshold, the quantum dot is considered “on” (emissive) and below it is considered “off” (non-emissive, dark). It is clear from the histogram that an “on” or “off” event does not correspond to a single on or off intensity but rather is a distribution of intensities above or below the assigned threshold.

![Figure 3](image)

**Figure 3.** (a) The red horizontal line indicates the assigned threshold. Signal above the threshold is taken to be an “on” event and signal below the
threshold is considered an “off” event. (b) Signal is plotted vs frequency in occurrence.

A probability distribution of fluorescence “on” or “off” events, which spans multiple orders of magnitude in probability, is shown in Figure 4. Current analysis of this data inherently places most emphasis on high-probability events, which results in erroneous interpretation of lower probability occurrences.

![Figure 4. Probability distribution of fluorescence “off” events. Data is fit to an inverse-power law, equation shown on graph.](image)

Using this technique, previous work has shown that fluorescence blinking statistics in CdSe/ZnS core/shell quantum dots is dependent on the excitation wavelength. Distribution results showed that the “on” statistics required an inverse-power law when excited on or near the band gap (575 nm). However, when the sample is irradiated around 250 meV above the band gap (525 nm), the “on” distributions are best described using a truncated power law. The truncated power law arises from decreased long-duration “on” events. More frequent cycling between on and off events was also observed. In contrast, the “off” statistics were not influenced by the exciting wavelength suggesting that the transition from an “off” to “on” event is not a photo-driven process. These findings are important for the implementation of quantum dot
based devices where performance can be significantly affected by the exciting wavelength.

Excitation-wavelength dependence has also been investigated in CdSe nanorods passivated with tetradecylphosphonic acid (TDPA). Nanorods also exhibit “on” power-law statistics when excited at the band gap energy, and a truncated power-law when excited 240 meV above the band gap. However, unlike the CdSe quantum dots, the “off” time statistics are also sensitive to the excitation wavelength, showing an inversely correlated effect to the “on” time statistics. In order to understand the influence of the environment in charge trapping, the particles were embedded in 1-ethyl-3-methylimidizolium bis(trifluoromethylsulfonul)imide room-temperature ionic liquid (RTIL) and compared to particles that were drop cast unto clean microscope coverslips. When the particles were excited near the band gap energy, no differences in blinking statistics were observed. However, when the particles were excited 240meV above the band gap, the nanorods embedded in the RTIL showed a decrease in the frequency of “on” events and more persistent “off” times. This suggests that a certain amount of energy above the band gap must be provided in order to access external and surface trap sites more readily which in turn is influenced by the embedding media.

This explanation follows from the charge carrier trapping model, one of the models that has been developed to describe the blinking behavior of fluorescent quantum dots. In this model, the quantum dots are believed to form charge-separated species when excited with light in which the electron may become trapped on the surface of the QD, preventing photon emission through non-radiative recombination and thus causing the observed dark states. It is possible that the RTIL embedding media, with a high dielectric constant, stabilizes the trap sites making “off” times persist over longer periods. However, the extent of the influence of embedding media on fluorescence statistics is currently unknown. Furthermore, the current microscope slide preparation for viewing the QD’s requires the polymer embedding media to be casted on the slide.
This method limits analysis to non-dynamic environments, which can be significant limitations in biological applications of single-molecule spectroscopy.

II. Aim of Study

Since absorption and fluorescence occur in $10^{-12}$ and $10^{-9}$ s, respectively, this study aims to investigate if the Pearson coefficient values of “on” events (fluorescence) are independent of frame rate. This is a necessary step for future application of the Pearson coefficient analysis on fluorescence intermittency of quantum dots exposed to different environments. By applying correlation studies across different frame rates we hope to gain a more fundamental understanding on the blinking statistics of “on” events. Once the influence of frame rate on correlation studies is fully understood, the Pearson coefficient analysis can be used in place of the probability distribution analysis to observe changes in the coefficient in response to environmental changes. Such experiments would give us insight into nanoparticle exciton relaxation dynamics (i.e. fluorescence and charge trapping) and the influence of these processes on nanoparticle optical properties.

III. Methodology

a. Instrumentation

A schematic of our current home-built microscope set-up is shown in Figure 5. The laser, a 532-nm solid state laser diode, is focused to the sample plane through the high numerical aperture (NA) microscope objective. The intensity of the laser is attenuated by a filter to 119.2 µW. The objective is a 100x Nikon Achromatic Finite Conjugate oil immersion objective with a 1.25 NA. The same objective collects the emission from the
sample attached to an x, y, z translation stage. The emission is directed through a long pass filter and imaged on an electron multiplying charge-coupled device (EMCCD), iXon Ultra 897, purchased from Andor Technology. Frame rates are adjusted using the Andor Solis software with a minimum of ~ 0.001 s acquisition time for our imaging area.

Figure 5. Schematic representation of single-molecule spectroscopy. M: Mirror; DM: Dichroic mirror; O: Objective; S: Sample; LPF: Long pass filter; CCD: Charge-coupled detector.

b. Sample Preparation

Coverslips The coverslips are first cleaned in a 3:1 concentrated Sulfuric Acid:Peroxide solution and sonicated for 30 minutes. Coverslips are removed from the solution and thoroughly rinsed with Millipore H$_2$O. They are submerged in Millipore H$_2$O and sonicated for another 30 minutes. The coverslip is then dried with nitrogen gas, rinsed with respective nanoparticle solvent, and dried again using the nitrogen gas.

CdSe nanoparticles The CdSe quantum dots used in this study were synthesized in the Knappenberger group. The bulk linear absorption and emission for these particles is shown in Figure 6. A dilute solution of CdSe particles is spin-coated onto a clean, 0.17 mm thick coverslip. The coverslip is then mounted on the translation stage and imaged.
Figure 6. Bulk linear absorption (black curve) and photoluminescence (PL) (red curve) of CdSe quantum dots. The laser excites at 532nm and the particle emits at around 570nm (approximately 150 meV above the band gap).

c. Pearson Correlation Coefficient

The Pearson’s correlation coefficient \( r \) measures the linear correlation between two variables and is calculated according to equation 1. In this study, this method is used to analyze the correlation between one “on” event \( x \) and the next “on” event \( y \). An \( r \) value equal to 1 indicates highly correlated data, \( r = 0 \) indicates uncorrelated data, and \( r = -1 \) indicates anticorrelated data.

\[
S_{xy} = \sum xy - \frac{\sum x \sum y}{n}
\]

\[
S_{xx} = \sum x^2 - \frac{(\sum x)^2}{n}
\]

\[
S_{yy} = \sum y^2 - \frac{(\sum y)^2}{n}
\]

\[
r = \frac{S_{xy}}{\sqrt{S_{xx}S_{yy}}}
\]

\(-1 \leq r \leq 1\)

IV. Results and Discussion

Images captured by the CCD camera of single nanoparticles are processed through ImageJ to obtain the fluorescence trajectories for frame rates of 200, 166.67, 133.33, 100,
66.67, 50, 33.33, and 25 s\(^{-1}\). A total of 234 different particles were imaged for a duration of 15 minutes across all frame rates. In order to study correlations in subsequent on-state durations, scatter plots of subsequent on-state duration versus on-state duration are created for each individual particle. Figure 7a is an example of highly correlated subsequent on-state durations for a single particle imaged at a frame rate of 166.67 s\(^{-1}\) with an r value of 0.65. Figure 7b represents a different particle imaged at 200 s\(^{-1}\) showing less correlated data with r = 0.24.

![Figure 7](image_url)

**Figure 7.** a) Correlation plot for a single CdSe nanoparticle showing highly correlated subsequent on events. Frame rate = 166.67 s\(^{-1}\), r = 0.65 b) Correlation plot for a different single CdSe nanoparticle showing poorly correlated subsequent on events. Frame rate = 200 s\(^{-1}\), r = 0.24.

This procedure was done for all 234 particles to obtain r values which were averaged and plotted versus frame rate, Figure 8. At frame rates below 70 s\(^{-1}\), average r values seem to drop faster than r values at higher frame rates where the data doesn’t change as drastically. This could be explained by the fact that since fluorescence occurs in 10\(^{-9}\) s and our fastest acquisition speed is 0.005 s, not all “on” events are “seen” by our instrumentation. Thus as the acquisition time gets faster the correlation is expected to increase. However, the large error bars show a high degree of variability in r values for different particles imaged at the same frame rate. In order to further investigate if the observed variation is due to inherent differences between particles in a single batch, the same particle was imaged for several different frame rates and is plotted in Figure 9.
Figure 8. $r$ values for 234 particles versus frame rate. All particles were imaged for 15 minutes using laser power of 119.2 µW. Errors bars are calculated to 95% confidence interval.

Figure 9. $r$ values show high degree of variability for a single particle across seven different frame rates.

Figure 9 shows high variability in $r$ values for the same particle across several different frame rates. This suggests that $r$ values obtained in future nanoparticle environmental condition studies may only be comparable for the same particle imaged.
at the same frame rate. However, further experimentation is needed to confirm that r values are consistent for the same particle imaged at a constant frame rate over time. This would ensure that an r value for a single particle obtained at a certain frame rate does not vary over time and any changes observed when the particle is subjected to different environmental conditions is in fact due to the environment. Extending this study to other particles imaged at other frame rates may show if data is in fact skewed at frame rates below 70s$^{-1}$.

V. Summary

In this study, the influence of frame rate on correlation studies of “on” events for single nanoparticles is investigated. Average r values for 234 particles imaged at different frame rates showed greater change in r value trends at frame rates below 70s$^{-1}$. Presence of large variability of r values within the same frame rate led to investigate r values for the same particle across all frame rates. This data also showed a high degree of variation in r values for the same particle across all frame rates. Overall, data suggests that (1) application of correlation analysis on future studies pertaining to the influence of environment on nanoparticle fluorescence will need to be done on the same particle and (2) r values may only be comparable for results obtained at the same frame rate for the same particle. Further experimentation is necessary to understand the full extent to which r values can be compared for a particle imaged at a constant frame rate. The data suggests that r values themselves may not be useful to analyze correlations between on events for a particle since they result in very different values depending on the frame rate chosen. However, a change in the r value for the same particle imaged at a certain frame rate under different environmental conditions may give some insight into changes in correlations of “on” events for a particle in different environments.
VI. Future Work

The next immediate step is to image the same particle at the same frame rate several times to confirm if the resulting $r$ value is constant. Once this is confirmed, this type of correlation study can be used to analyze data such as that shown in Figure 10. Using the binary set analysis method, the probability distribution curve for on events at pH 7 appears to be truncated in comparison to the probability distribution curve for pH 9. From this preliminary data, it appears that the surrounding pH influences the fluorescence behavior of these particles. We hope to implement correlation studies for such data to gain better insight into relaxation exciton dynamics than the current probability distribution analysis provides.

![Figure 10. a) Probability distribution curve for single CdSe nanoparticle submerged in water of pH 7. b) Probability distribution of a different particle submerged in water of pH 9. This preliminary data suggests the blinking statistics of these nanoparticles are influenced by the environmental pH.](image-url)
VII. References


