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3D Single Particle Tracking Spectroscopy and Nanoparticles for Interrogating Complex Systems

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3D Single Particle Tracking Spectroscopy and Nanoparticles for Interrogating Complex Systems

by

Daniel Montiel

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate Division of the University of California, Berkeley

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Professor Richard A. Mathies
Professor Thomas C. Alber

Fall 2012
3D Single Particle Tracking Spectroscopy and Nanoparticles for Interrogating Complex Systems

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Daniel Montiel
Abstract

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Daniel Montiel

Doctor of Philosophy in Chemistry
University of California, Berkeley
Associate Professor Haw Yang, Chair

3D single particle tracking spectroscopy is a technique which allows a person to follow a freely diffusing nanometer-sized particle in three dimensions, in real-time. The basic scheme involves illuminating a nanometer-sized probe with a confocal microscope, optically detecting its position change with avalanche photodiodes, and then moving a piezo-electric stage to counteract the particle’s movement. The tracked particle is centered within the collection volume of a confocal microscope, enabling concurrent spectroscopic readouts of the tracked particle’s emission.

The motivation for this technique is the desire to perform spectroscopy in complex environments. Dynamics in complex environments, such as within a living cell, occur over a range of time and length scales. Unlike homogeneous systems, the multiple dynamical length and time scales are correlated, prohibiting the use of simplifications like timescale separation. In order to understand the correlations across different scales, new experimental techniques are needed which can provide long-time observation and spatially-correlated dynamical measurements. 3D single particle tracking spectroscopy is the realization of this dream for nano-scale environments. Counteracting the particle’s diffusion with the piezo stage enables long-time observation of the freely diffusing particle. Because the current stage position is a measure of the particle’s position, one can perform spatially-correlated spectroscopy.

The scientific impact of this thesis has been to develop the 3D single particle tracking spectroscopy technique, demonstrate the potential of using nanoparticles to design new kinds of single-molecule/probe assays, and open the possibility of using an under-appreciated class of bright, photo-stable nanoparticles (large gold nanoparticles) for biophysical applications.
To Grandma and Grandpa
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6.1 Normalized excitation and emission spectrum of 350nm type Ia diamond nanoparticles obtained with SpectraMax M5 (Molecular Devices) spectrometer. Above the spectrum, there are three schematic drawing represent different color centers, N3, H3 and (N-V)- centers. The filled circles (black) represent nitrogen; filled circles with grey color are carbon, and open circles represent vacancies. The dotted line (marked with ∆) to the left is the excitation spectrum of H3 center, and the solid line marked with ∆ is the emission spectrum of H3 center. From the H3 center emission spectrum, the peak wavelength is around 530nm, and the excitation scan was obtained by fixing the emission wavelength at 530nm, the obtained excitation peak wavelength is around 450nm. The arrow pointed at 405nm illustrates the wavelength position of the excitation wavelength of our laser system. 530nm with an additional broad band spanning from 600-800nm contributed by the (N-V)- centers. The dotted line (marked with open circle) in the middle is the excitation spectrum of (N-V)- center, and the solid line marked with open circle is the emission spectrum of (N-V)- center. From the (N-V)- center emission spectrum, the peak wavelength is around 650nm, and the excitation scan was obtained by fixing the emission wavelength at 650nm, the obtained excitation peak wavelength is around 550nm.

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7.1 The chemical structure of dihydrolipoic acid - sulfobetaine (DHLA-SBE). Reprinted with permission. Copyright Springer.

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Acknowledgments

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Chapter 1

Introduction

1.1 Complex Systems

Emergence is one of the defining characteristics of a complex system. Generally, emergence is used to describe phenomena arising from a system which could not have been predicted without a priori knowledge just given the elements of a system. It arises in variety of fields in different contexts.

In mathematics, as one randomly connects nodes and vertices in a graph the largest number of connected nodes will scale with a power-law dependence [1, 2]. Above a certain threshold number of connections, there can exist a phase transition whereby a graph of many small disjointed nodes transitions to one of a few large connected ones [3]. This seemingly abstract concept has important real-world implications. The emergence of percolation underpins the fault tolerance of the internet [4, 5] and epidemiology models [6].

In the material sciences field, glasses are a physical kind of complex system. Microscopically they are locally disordered like liquids even though macroscopically their inability to flow makes them structurally rigid like solids [7]. The structural rigidity and bulk viscosity emerge from domains of heterogeneous microscopic dynamics with long range correlations. An enormous amount of experimental and theoretical research has been conducted to understand this complexity [7–11].

Similar to glassy systems, biological systems can be also be viewed as a type of complex system resulting from long range correlations of local dynamical heterogeneity. Biological phenomena occur over a range of spatial and temporal length scales, from picosecond electron transfer processes [12], to nanosecond protein side-chain rotation [13], to μs and ms protein conformational dynamics [14–16], to the many orders of magnitudes over which proteins fold [17]. In neurobiology for example, scientists try to understand how consciousness emerges from the collective interaction of the millions of neurons in our brains [18]. Just like glassy systems, the phenomena in neurobiology span multiple different length scales from the single cell level [19] to the whole connectome [20]. Biological systems display temporal complexity as well. Higher organisms start out as single fertilized embryos. As cells grow and divide,
tissue differentiation occurs even though all cells contain the same genetic information. The collective interactions of all the intra- and inter-cellular processes breaks the symmetry of the initial conditions [21]. Understanding these biological phenomena requires rejecting simple separation of timescale dynamical arguments because the inter-related timescales span the entire life cycle of the organism. New experimental tools are needed in order to understand and measure these multi-scale interactions in context.

1.2 Nanoscale Environments of Complex Systems

When the concept of temperature is first introduced in an undergraduate curriculum it is usually discussed in terms of a water bath model. Increasing temperature imparts more kinetic energy to solute molecules in the bath from the increased Brownian motion of the solvent molecules. The bath is implicitly described to be infinitely large and homogeneous. Within a single cell, these fundamental assumptions break down. It has been shown experimentally that different regions of the cell are at different temperatures [22]. At the single protein level, increasing temperature can result in altered enzymatic activity levels [23]. Within a whole cell, environmental stresses such as temperature can alter protein expression levels [24–26]. At the whole organism level, hypothermia, for example, has been shown to reduce neural function after cardiac arrest [27]. This simple concept of temperature spans different biological length scales in diverse related contexts. The cell at any particular time is out of equilibrium due to perturbations of different environmental stresses. Because intracellular environments are heterogeneous, crowded and spatially confined, one might expect the kinetics from an in-vitro biochemical assay to differ from the true in-vivo behavior as the underlying equilibrium assumptions breakdown [28–30]. Using dextran as a viscosogen to mimic cellular crowding has shown the rate of amyloid fibril formation can be altered [31], demonstrating that the context of the local environment can impact biological activity.

The crowded intracellular environment is also spatially segmented into regions performing different functions. Genes are transcribed in the nucleus, translated at the ribosomes, and nascently folded within the endoplasmic reticulum [32]. Once folded, different proteins can intercalate within membranes [26] or become localized at other organelles such as the mitochondria [33]. These different local environments of pH, chemical concentration, local viscosity, etc., lead to changes in dynamical behavior across various biological length scales in the same way that changes in temperature span scales from the molecular to whole organism level. Techniques which can span various length and time scales can provide insight into how the local intracellular environment impacts dynamics across multiple scales.

1.3 Nanoprobes of Local Environment

Nanoparticles are tools which can probe local, biologically-relevant length-scale environments. Different classes of nanoparticles such as quantum dots, metallic nanoparticles and
fluorescent molecules each have their own advantages and disadvantages for probing local environments.

Quantum dots are small inorganic crystals typically made with a band-gap semiconductor [34, 35]. When optically excited, an electron-hole pair is generated which can radiatively recombine [35]. When the length dimensions of the crystal are on the order of a few nanometers, the wave-function describing the electron and hole become quantum confined leading to size dependent optical emission with a large Stokes shift [35]. Their emission can be tuned across the visible and near-infrared regimes [36], useful for in-vivo applications [37]. The utility of quantum dots for studying complex systems is their environment dependent emission properties. The bandgaps of semiconductors are known to be sensitive to temperature, resulting in a spectral red-shift with increasing temperature [38]. Various sensing quantum dots, including pH [39], redox state [40], electric field [41], and water-soluble ratiometric temperature, have also been synthesized [42, 43]. Their known surface chemistry allows them to be easily conjugated to biological systems [44, 45]. These advantageous properties have made them useful for probing intracellular environments.

One of the disadvantages of using quantum dots is their well known blinking behavior [46-49]. The luminescence emission from quantum dots display intermittent intensity levels alternating between bright and dark states. This behavior is thought to be related to charging on the quantum dots, but is still an active area of research. To prevent this behavior, they are typically overcoated with a large bandgap material to prevent build-up of charges on the core [46, 49]. Another problem with quantum dots is photo-oxidation of the core. The quantum dot core can irreversibly oxidize leading to blue-shifted emission and decreased emission intensity [50]. Overcoating the core quantum dot layer seems to mitigate both the blinking and photo-oxidation behavior [51].

Gold nanoparticles are both bright and optically stable. It has been known since antiquity
that metallic nanoparticles give rise to various optical properties [52]. The collective oscillations of surface electrons in metallic nanoparticles move in response to an applied electric field, termed the plasmon resonance [53, 54]. This phenomena is strongly size dependent and the relationship for spherical particles was first described by Gustav Mie in 1908 [55]. The emission from gold nanoparticles is derived from scattering and absorption contributions. As seen in Figure 1.1, the extinction spectra can be tuned by changing the size, shape and material of the nanoparticle. Above 60 nm in diameter, scattering becomes the dominant factor making those particles easily visualized by darkfield microscopy.

Besides particle size, the plasmon resonance is sensitive to the near-field dielectric environment [53, 54]. Adsorption of molecules to the particle’s surface can cause a red-shifting of the particle’s plasmon resonance. This property has been used for analyte detection in sensing type applications [56]. Because large gold nanoparticles can be easily visualized by dark field scattering [57], they have also found imaging and tracking applications for biophysical studies. They also have beneficial properties such as being photostable, bright and also remotely heatable [58, 59].

Fluorescent molecules are another useful class of probe studying local environment. Because of great synthetic control, the emission wavelength of fluorophores can be tuned across the visible spectrum. They can be designed to respond to changes in pH [39], temperature [60], electric field [61], peroxide concentration [62], and enzyme activity [63, 64] among other properties. Fluorescent molecules blink and are also limited by the number of photons that can be collected before irreversibly photo-bleaching [65]. The blinking in fluorescent molecules is a result of a transition to a dark triplet state. The photo-bleaching results when the excited singlet state reacts with triplet oxygen to form a non-emissive species [66].

Nanoparticles not only allow for the study of environment-correlated dynamics, they also can provide long-time observation as well. Large gold nanoparticles are optically stable and can be conjugated to either biological systems or other kinds of environmental sensors. 3D single particle tracking spectroscopy of nanoparticles therefore acts as a general platform for dynamical characterization across various time and length scales using these kinds of hierarchical particle systems.

1.4 Summary

3D single particle tracking spectroscopy is an experimental technique enabling the long-time and spatially-correlated spectroscopic characterization of complex environments. This thesis describes the 3D single particle tracking technique, theoretical developments for data analysis, characterization of various nanoprobes and the robust conjugation of biological and inorganic nanomaterials. The contents of the chapters are based on previously published works [67–69], a recently accepted book chapter, and one unpublished manuscript. Chapter 2 reviews the principles of 3D single particle tracking. Chapter 3 describes the use of a gaussian changepoint analysis to look for changes in diffusive behavior. Chapter 4-6 describe studies to characterize the optical properties of gold nanoparticles, quantum
dots, and Type Ia nanodiamonds. Chapter 7 describes robust passivation procedures which have been developed in order to interface inorganic nanoparticles with biological molecules. Chapter 8 discusses some of the future directions based on this thesis work.
Chapter 2

Real-time three-dimensional single-particle tracking spectroscopy for complex systems

2.1 Abstract

Complex systems are characterized by dynamical processes spread over multiple time and length scales. At a given instant, these systems can display spatial heterogeneities in which the local physical and chemical properties are non-uniform, depending on the location. They can also exhibit dynamical heterogeneities in which the local dynamical characteristics vary with time. These types of systems pose unique experimental challenges for their characterization and test of theoretical ideas. Recently, real-time three-dimensional (3D) single-particle tracking spectroscopy has been developed to address these kinds of problems. With this approach, in principle, one can follow how a system evolves spatially as well as temporally. This article attempts to provide an introduction to this promising new technique by discussing the aims of studying of complex systems and recent experimental advances towards this goal.

2.2 Introduction

The term “complex system” has come to typically refer to systems composed of many components whose collective interactions lead to unexpected properties and phenomenon. Examples of these kinds of systems appear in nearly every scientific discipline. Given its breadth and diversity, it is not the intent of this review to summarize all areas of this subject. Rather, here one considers the physical chemistry and chemical physics of a physical system (as opposed to abstract theoretical objects) whose microscopic dynamics evolve with time and position over many orders of magnitude a subset of the more generally defined “complex systems.” These physical systems are challenging to study because conventional
experimental tools designed for simple systems are insufficient to unravel the details of the underlying processes; indeed, the lack of experimental information could also impede conceptual advances. The aim of this overview is to discuss a promising experimental technique, real-time three-dimensional single-particle tracking spectroscopy, with the potential to characterize and follow the local activities of a system over multiple time and length scales.

Understanding the nature and evolution of the microscopic interactions is central to many endeavors in a variety of interrelated areas in physical sciences. In materials science for example, one would like to clarify how the dynamics in local domains of super-cooled liquids near the glass transition relate to observed bulk properties such as viscosity [7–11]. In applied physics, understanding the rules of nanoscale self-assembly will in principle help guide bottom-up approaches to reliably create complicated functional nanostructures with length scales extending to micrometers and millimeters with minimal defects [70]. In chemistry, when the timescale of a molecular reaction overlaps with the timescale of molecular relaxation, the chemical reactivity remains poorly understood, because the powerful principles of conventional chemical kinetics were built upon equilibrium and near-equilibrium statistical mechanics [71]. Analogously, in biochemistry and molecular biology, the timescale on which biological macromolecules assemble to form a multi-subunit complex can overlap with the timescale of subunits conformational dynamics. More generally, a fundamental question is whether the biochemical insights obtained through well-controlled in vitro experiments directly reflect what happens in highly heterogeneous and localized compartments in cellular environments [28–30].

Figure 2.1 illustrates on a more abstract level the spatial-temporal correlation of local properties in a complex system. In contrast to simple systems, the local property of a complex system can either be location-dependent or time-dependent, or both. Experimental techniques designed for simple systems are inadequate because, for example, they cannot distinguish scenario (b), static heterogeneity, from scenario (c), dynamical heterogeneity, as depicted in Figure 2.1. In fact, in most cases, one would expect a scenario depicted by Figure 2.1d with convoluted static and dynamical heterogeneities. Therefore, new experimental tools need to be developed in order to provide correlated time- and position-dependent information of a microscopic system.

### 2.3 Local Dynamics of a complex system

In order to formulate a conceptual framework for macroscopic phenomena from a microscopic model, one must carefully consider the relevant local interactions. To get a better idea for the kind of information needed from experiments, several examples along the lines of the previous section are given below. Also pointed out in each example are representative key questions not readily addressable using currently available technologies. The purpose of the present discussion is not to provide a thorough review of the various fields; rather, the intent is to give the readers a sense of the necessity for studying complex systems and the need for new experimental techniques to tackle these problems.
Figure 2.1: Caricatures illustrating spatial-temporal correlation of local properties in complex systems. In all panels, $\vec{p} = (p_1, p_2, \ldots, p_n)$ represents the local property vector, either dynamical in origin (relaxation rate, diffusion coefficient, viscoelastic response, and so forth) or static (pH, structural configuration, molecular conformation, etc). $\vec{q} = (x, y, z)$ represents the three-dimensional coordinate vector; and $t$ is time. The time and length scales accessible to conventional ensemble-averaged experiments are denoted by $\Delta_t$ and $\Delta_q$, respectively. (a) In simple systems the local properties are constant within the experimental period and are not dependent on the location. (b) In systems showing “static heterogeneity,” the local properties do not change over time, but depend on the location. (c) In systems that exhibit “dynamical heterogeneity,” the local environment of a probe does not depend on the location but varies with time on a timescale extending beyond typical ensemble-averaged experimental methods. (d) In general, the properties of a micro-domain in a complex system may evolve with time and the micro-domain may migrate across compartmentalized spatial regions with distinct physical and chemical environments; such microscopic dynamics lead to the macroscopic behavior of the complex system. Reprinted with permission. Copyright John Wiley and Sons.
CHAPTER 2. REAL-TIME THREE-DIMENSIONAL SINGLE-PARTICLE TRACKING SPECTROSCOPY FOR COMPLEX SYSTEMS

For a complex system composed of heterogeneous micro domains, the relaxation time scales within these domains may persist longer than the timescale of rotational and translational diffusion over the domain length scale. These micro domains are characterized by their distinct relaxation dynamics even for systems composed of a single chemical component. The size of each micro domain can be as small as a single molecule, on the order of few nanometers. A representative case is super-cooled molecular glass forming materials. Near the glass-transition temperature, the picture of spatially heterogeneous dynamics is supported by both ensemble-averaged experiments [72–74] and single-molecule investigations [75–77]. However, there remain many questions before a full picture can be established. Questions stemming from the micro heterogeneity picture (cf. Figure 2.2a) include whether the micro-domains migrate on a longer-term timescale and if so, is the mode of migration diffusive. Another question along the lines of dynamical heterogeneities is whether the mode of relaxation dynamics within the same micro domain evolves over time and if so, what are the characteristic dwell times in each relaxation mode. Answers to these questions are essential in building a fundamental physical picture and will be instrumental in testing different theoretical models.

In the spontaneous assembly of nanostructures, molecular aggregates, and biological complexes, the structure of an assembly tends to evolve over many different forms of approximately equal free energy while it seeks the most favorable configuration. Key questions in the self-assembly process revolve around understanding the distribution of different meta-stable configurations and how they affect the observed macroscopic rate of formation of the final structure (cf. Figure 2.2b). In addition to the problem of lacking suitable tools to study the meta-stable transient structures in situ, there are also experimental challenges in studying the dynamics. For example, one is interested to know the timescale as well as pathway of relaxation associated with each meta-stable configuration. Any subunit within the assembly will experience cooperative interactions from other subunits. In many cases, the timescale of such a structural relaxation, considered part of the overall chemical transformation process, overlaps with transportation of components to the assembly. Analogously, the conformational relaxation timescale of an enzyme can range from microseconds to hundreds of seconds under physiological conditions, overlapping with the timescale of typical catalytic cycles [12, 16, 81, 84, 85] (cf. Figure 2.2c). In both the self-assembly and the protein dynamics cases, even when the constituting units in a nano-assembly are well characterized and the structure of an enzyme is well established, one still cannot predict the mechanism of assembly or the rate of enzymatic catalysis. These considerations are to be contrasted with small-molecule reactions in solution where the structural relaxation timescale is on the order of picoseconds whereas the molecular transport timescale is on the order of nanoseconds. When considering small-molecule reactions either experimentally or theoretically, the treatments are drastically simplified by exploiting ideas of separation of timescales a luxury not available to studies of complex systems.

A still more complicated case is the biochemical processes inside a living cell. The interior of a cell is crowded with an assortment of biological macromolecules. Furthermore, the intracellular environment is highly heterogeneous and, for eukaryotic cells, is divided
Figure 2.2: Example cases that are amenable to investigations using real-time 3D single-particle tracking spectroscopy. (a) Spatially heterogeneous dynamics of super-cooled liquids near the glass transition temperature. The micro domain may evolve with time. Illustration was adapted from Figure 2b in Ref. [10]. (b) In self-assembly, it may have to go through many equal-energy intermediates before the configuration reaches the desired final structure. The timescale for the reconfiguration is comparable to that of subunit diffusion. (c) The timescale of protein dynamics is comparable to those of substrate diffusion and catalysis. (d) Free energy as a function of configuration coordinate, collectively sampled over all coordinate space at a given structural configuration. Such a landscape picture, though averaged over local structures and omitting dynamics, has been successfully used to describe many qualitative features in glass transition [78, 79], protein folding [80], and protein dynamics [81–83]. Reprinted with permission. Copyright John Wiley and Sons.
into different compartments containing different chemical environments such as pH, as well as physical states such as viscoelastic response, and possibly even temperature. Knowledge of the nanoscale physical and chemical properties is crucial for translating the biochemical insights obtained from in vitro characterizations into a realistic cellular setting.

The above discussions illustrate the importance of a quantitative understanding of local environments and the dynamics within. A necessary requirement for the study of complex systems such as those listed above is therefore the ability to characterize the system on multiple time and length scales. New experimental tools are expected to help resolve these outstanding questions and invigorate many interrelated fields, old and new.

The investigation of molecule-scale local properties in a spatially heterogeneous environment has been made possible with the advent of single-molecule spectroscopy [86, 87]. Furthermore, the concurrent rapid advances in commercial detector technology have significantly reduced the barrier towards achieving this goal. To allow the detection of single molecules or single nanometer particles, the use of optical probes with suitable properties is important. Here, except for specific examples, the generic term “nanoprobe” will be used to categorically represent optical probes that afford single-particle or single-molecule spatial resolution and are smaller than the diffraction limit (300 nm in diameter). Recently, several methodological advances have been made towards characterizing a nanoprobe’s local micro environment. For example, Li et al. have shown how individual semiconducting quantum dots can be used to sense the local temperature [88]; Xu et al. have advanced methods to accurately measure the energy transfer between a blinking quantum dot and a fluorescent dye [89, 90], a particularly advantageous class of pH sensing probe allowing for long-term monitoring.

Dynamical properties such as the viscoelastic response and its time-dependent changes can be measured by following in real time the rotational and translation displacements of a nanoscale probe for prolonged periods. Experimentally, this can be achieved using particle tracking, but high time resolution will be needed in order to study local dynamics. For example, one sensitive type of measurement of the micro-domain viscoelastic response is the rotational diffusion of an optical nanoprobe. The sensitivity and hence discrimination power comes from the fact that the rotational diffusion timescale is proportional to the cube of a particle radius. In other words, it will require a time resolution of at least 10 µs for using a 100-nm diameter spherical nanoprobe; even better time resolution will be required for experiments studying smaller domains with smaller probes.

Real-time single-particle tracking combined with optical spectroscopy, while technically extremely challenging, can be a powerful tool to study complex systems by combining the advantages of optical spectroscopy with the ability to follow the probes position over time, where the local physical and chemical state can be studied as a function of the probes position.
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2.4 Real-time 3D single-particle tracking spectroscopy

Single-particle tracking, broadly, is the study of physical, chemical, and biological processes from the analysis of an optically detected probe particle’s trajectory. Particle tracking through imaging and off-line image analysis is a relatively well developed field, for which there have been several excellent reviews for applications in biophysics [91], soft condensed-matter physics [92], and many others. The basic type of data from this kind of experiments is the probe particles’ positions as a function of time, or trajectory. For example, for sufficiently long trajectories, the probe particle’s transport mode can be uncovered through statistical analysis of the data [93, 94],

\[ \langle |\Delta r|^2 \rangle = \langle (\mathbf{r} - \langle \mathbf{r} \rangle)^2 \rangle = 2dK_\alpha t^\alpha \]

with

\[
\begin{align*}
0 &< \alpha < 1, \text{sub-diffusive;} \\
\alpha &= 1, \text{normal diffusion (Brownian motion);} \\
1 &< \alpha < 2, \text{super-diffusive;} \\
\alpha &= 2, \text{ballistic limit;} \\
\alpha &> 2, \text{turbulence,}
\end{align*}
\]

(2.1)

where \( \langle \cdots \rangle \) represents ensemble averaging or time averaging over a period of \( t \to \infty \), \( \mathbf{r} \) is the particles position, \( d \) is the dimensionality, and \( K_\alpha \) is the diffusion coefficient. Under favorable conditions, furthermore, the viscoelastic response of a probe particles local medium can be extracted from its trajectory, providing insights about the microscopic interaction between the probe particle and its environment. The complex shear modulus, \( G^*(\omega) \), for a spherical particle can be calculated from the generalized Stokes-Einstein relationship in the Fourier space [95, 96],

\[ G^*_{r}(\omega) = \frac{k_BT}{i\omega a \langle |\Delta r(\omega)|^2 \rangle}, \]  

(2.2)

where \( \omega \) is the angular frequency, \( a \) is the radius of the particle, \( k_B \) is the Boltzmann constant, and \( T \) temperature. The elastic and viscous moduli are given by the real and imaginary parts of \( G^*(\omega) \), respectively. Similarly, the local viscoelastic response can also be measured via rotational motions using [97, 98],

\[ G^*_\theta(\omega) = \frac{k_BT}{i\omega a^3 \langle |\Delta \theta(\omega)|^2 \rangle}, \]  

(2.3)

where \( \langle |\Delta \theta(\omega)|^2 \rangle \) is the frequency response of the mean squared angular displacement.

It is important to note, however, the length of a trajectory in practice is usually limited; more advanced statistical treatments are necessary to interpret the data correctly [99]. Dissecting the time dependent changes in the translational [67] and rotational [100] dynamics of the probe particle can potentially give information about changes in the underlying

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transport mechanism, as well as information about changes in the local environment. More recently, finite 3D resolving capability has been demonstrated on the single-molecule level by analyzing the emission pattern of defocused images [101–103], similar to ideas used widely in the compact disk (CD) industry [104].

Real-time 3D particle tracking goes beyond the conventional imaging-based single particle tracking by resolving the particles three-dimensional displacement during the experiment (as opposed to off-line image analysis), and uses that information to counter the particle’s movements in real time. Such a closed-loop feedback mechanism allows for the long-term observation needed for following the particle’s time-dependent changes, fast and slow. More importantly, it allows one to carry out spectroscopic studies at the same time. The earliest demonstration of closed-loop 3D single-particle tracking appears to be the work by Berg [105], who was able to successfully track micron sized bacteria in three dimensions. In Berg’s scheme, the axial Z displacements were decoded using two optical fibers off the image plane, and the in-plane X-Y movements were decided using a 4-fiber bundle in a quadrant configuration. However, real-time single-particle tracking of nanoprobe has been difficult. Firstly, it requires resolving target displacements smaller than the size of optical diffraction. Secondly, the nanometer size of the probe implies high translational diffusion mobility, requiring a very fast feedback response mechanism. It was not until the advent of commercially available fast piezoelectric stages and field-programmable gate arrays has it been possible to successfully carry out real-time 3D tracking of nanometer-sized materials. It ought to be pointed out that “real-time” is a relative term; here, it is defined operationally as the timescale of probe transport, be it diffusive or active, on which the probe is always kept within the optical detection focal volume. An overview of the various technical aspects for real-time 3D single-particle tracking has appeared recently [106]; therefore, they are not repeated and only the principles are briefly outlined below for completeness.

2.5 Experimental Implementations

Resolving the axial displacement of a nanoprobe is a major challenge in 3D single-particle tracking. One of the basic principles circumventing this difficulty is the realization that the 3D intensity distribution at the image focal point encodes the axial as well as the lateral position information. In other words, one should be able to resolve the 3D positioning of a probe particle with nanometer resolution through suitable analysis of the three-dimensional intensity pattern (cf. Figure 2.3a). To a very good approximation, a nanometer-scale emitter at the object focal point will project an intensity pattern described by a Gaussian profile at the image focal point [54]. The probe particle’s displacement can then be recovered by analyzing changes in the mean position of this intensity profile. This scheme relies on having only one light emitting source at the object focal point, projecting a well-defined diffraction pattern at the image focal point [54]. The probe particle’s displacement can then be recovered by analyzing changes in the mean position of this intensity profile. This scheme relies on having only one light emitting source at the object focal point, projecting a well-defined diffraction pattern at the image focal point configuration akin to that in confocal microscopy. Therefore, this scheme is called the confocal tracking method and was first demonstrated for tracking nanometer-size particles in 3D by Cang et al. [107]. In that initial experiment,
Figure 2.3: Sensing 3D positioning in real time with nanometer resolution. (a) Schematic illustration of how a nanoprobe-projected diffraction pattern at the image focal plane encodes the three-dimensional (3D) position information of the probe. (b) Decoding the 3D position of a nanoprobe under a confocal configuration using an overfilled pinhole to resolve the axial position and prism pairs for the lateral displacements. The inset shows a modern implementation of the Berg design using axially offset X and Y fiber pairs to resolve the Z position. (c) Basic principles of the scanning demodulation design showing the modulation signal when the probe is at the center of the objective lens focus (top panel), at the object plane but off-centered (bottom-left panel), and displaced from the objective center both axially and laterally (bottom-right panel). (b) and (c) were reproduced with permission from Ref. [106] with modifications in the layout, Copyright Elsevier.
a quadrant avalanche photo-diode (APD) was used to sense the lateral displacement. In an alternative implementation pairs of reflective prisms in the conjugate image plane were used for lateral X-Y displacements [108] to allow the use of single-photon counting APD for single-molecule sensitivity (Figure 2.3b). In both implementations, the axial Z position was decoded by sensing the intensity gradient along the axial direction using an overfilled pinhole. More recently, Lessard et al. implemented a real-time 3D single-particle tracking system using pairs of misaligned optical fibers in a similar but elegantly simplified implementation of Berg’s design [109]. In all these different confocal implementations to date, the creation of an intensity gradient has been used to retrieve the axial information.

The other approach is the fluorescence demodulation technique, which relies on the principle of confocal scanning. It was first theorized by Enderlein [110], and experimentally demonstrated by Levi et al. [111] and later by Berglund et al. [112] (Figure 2.3c). By laterally scanning very rapidly the excitation volume of a tightly focused laser around the probe particle of interest, the intensity of the collected fluorescence will be modulated by the particle distance from the focus, whereas the phase of the fluorescence intensity with respect to the lasers position will indicate the direction. In order to break the axial symmetry, one must perform two scans, once above and once below the primary image plane. In practice, the laser focus is offset from the plane of the tracked particle in order to increase the illuminated area of the excitation light. The restorative feedback mechanism in this configuration can be either repositioning the microscope objective lens, or driving a piezoelectric stage on which the sample rests. In both the confocal tracking and fluorescence demodulation implementations, the positioning information from detector output is not recorded but used for real-time feedback control by moving the entire sample to an opposite direction with same magnitude as the nanoprobe displacement. The time-dependent location of the probe comes from the nanometer-precision capacitive sensors, now standard in most commercial piezoelectric stages.

Although it is exciting to be able to track single particles in three dimensions, it is not enough. To understand the viscoelastic microenvironment of a nanoscale probe and how it changes with time, one will need to measure the rotational and translational diffusion dynamics rapidly and reliably. To determine the local temperature and pH on the nanometer scale, it is necessary to record spatially correlated spectral information from a moving nanoprobe. The next section describes proof-of-principle experiments demonstrating these capabilities, thereby putting forward the real-time 3D single-particle tracking spectroscopy as a viable new tool ready to tackle questions in complex systems.

2.6 Proof-of-principle demonstrations for measuring key parameters on the nanometer scale

Translational diffusion of fluorescent nanoparticles. For many applications, especially in turbid medium, fluorescence or other luminescence is the detection mechanism of choice.
Both the fluorescence demodulation approach [111, 113] and the confocal tracking method [108, 109] have been shown to deliver reasonably robust measurements of the translational diffusion coefficient. Levi et al. did one of the earliest intra-cellular applications of real-time closed-loop 3D single-particle tracking experiments using two-photon excitation to follow phagocytosis of protein-coated fluorescent beads by fibroblast cells [111]. An example of the new information that can be learned is given by the subsequent 2D real-time closed-loop tracking experiments by Levi et al.. They tracked fluorescently labeled chromatin on two dimensions and reported that the particles appeared to undergo free diffusion interrupted by jumps of approximately 150 nm [114, 115]. Although the biological significance of the observed changes in diffusive behavior requires further study, it nevertheless illustrates the power of real-time single-particle tracking; the high temporal (32 ms [114]) and spatial (20 nm) resolution allowed for the observation of new phenomena.

Translational diffusion of non-fluorescent nanoparticles. For applications with materials that are non-fluorescent, Xu et al. have shown closed-loop tracking that can be applied to following the diffusion dynamics of gold nanoparticles in water (diameter = 80–250 nm, corresponding to diffusion coefficients of 6.13 ± 1.96 µm²/s, respectively) using a dark-field illumination configuration [90]. With a time resolution of 100 µs and spatial localization of
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Figure 2.5: Anti-bunching from a single quantum dot freely diffusing in water (diffusion coefficient $20 \mu m^2/s$). Figure reprinted with permission from Ref. [116], Copyright 2007 American Chemical Society.

16 nm, they were able to quantitatively and rapidly recover accurate diffusion coefficients. Moreover, comparison with the size distributions of these nanoparticle samples from transmission electron microscopy to the distributions from tracking showed the ability to recover the distribution as well as the mean diffusion coefficients (Figure 2.4).

Concurrent spectroscopic measurements. An important goal for real-time closed-loop particle tracking is the ability to follow time-dependent changes in spectroscopic observables and correlate them with spatial location of the probe in order to better understand the system’s microscopic local environments [107]. The first step to accomplishing this goal is necessarily the ability to do spectroscopy on the tracked nanoscale particle. Using the confocal tracking technique, Cang et al. were able to record the scattering spectra of tracked freely diffusing particles [106]. More recently, Wells et al. characterized anti-bunching photon statistics from freely moving single quantum dots but under high background conditions similar to cellular conditions [117]. Using the fluorescence demodulation approach, McHale et al. were able to observe anti-bunching in tracked quantum dots diffusing in water with a diffusion coefficient of $20 \mu m^2/s$ [116] (Figure 2.5). Being able to measure anti-bunching photon statistics from a single quantum dot while tracking is important because it allows one to unambiguously assign the number of quantum dots being tracked rather than making a claim based on visual inspection of the blinking behavior as has been done in the past.

Rotational diffusion. Compared with translational diffusion, following the rotational diffusion dynamics of a single nanoprobe in real time is a much more demanding task, requiring the capability of performing time-dependent spectroscopy on tracked particles. For example, for probes with a well-defined emission dipole such as single molecules [118–121] or single non-spherical quantum dots [68, 122, 123], this can be achieved in principle by measuring the
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Figure 2.6: Directly following rotational diffusion of single gold nanoparticles through plasmon scattering spectral fluctuations (adapted from Ref. [106] with permission, Copyright American Institute of Physics). (a)(d) Conceptual drawing for the angle-dependent plasmon scattering. (e) Spectral correlation time (rotational diffusion) as a function of nanoparticle hydrodynamic radius inferred from translational diffusion of sizes 80 nm (green), 100 nm (red), 150 nm (blue), and 200 nm (black). The inset shows representative spectral fluctuation relaxation traces using the same color codes. (f) Polarization correlation time (rotational diffusion) as a function of nanoparticle hydrodynamic radius inferred from translational diffusion. The color codes are the same as in (e). The inset shows a representative 3D trajectory. The shaded regions in (e) and (f) corresponds to 15% deviation from the Debye-Stokes-Einstein equation, $\tau_{\text{relaxation}} = \frac{\pi \eta a^3}{6 k_B T}$, where $\eta$ is the bulk shear viscosity, $a$ is the particle radius, $k_B$ is Boltzmann constant, $T$ is temperature. In order to capture rotational diffusion dynamics, the time resolution for this experiment was pushed to 10 $\mu$s.
emission polarization anisotropy. It is not apparent, however, how to approach this problem for non-fluorescent nanoprobes such as metallic nanoparticles. By directly following the time-dependent changes of scattering spectrum from individually tracked gold nanoparticles (cf. Figure 2.6), Cang et al. found, surprisingly, the spectrum fluctuates with time [106]. They were able to correlate the spectral fluctuation to the rotational diffusion of those tracked particles [124]. Since the scattering spectra from a nanometer-size cluster depends sensitively on the orientation and the shape of the cluster [125, 126], this experiment has the implication of using real-time closed-loop single-particle tracking to study self-assembly problems. Furthermore, the capability of accurately and rapidly determining equilibrium transport coefficients holds the prospect of utilizing diffusion as a dynamic observable.

Guided imaging. With appropriate probes, it is now within reach to follow the local dynamics for a prolonged period, addressing the problem of multiple time scale in a complex system. For the multiple length scale problem, the notion of “guided imaging” provides a tangible solution [108]. The idea is that at the probe location and its vicinity, one desires high spatial and temporal resolution for spectroscopic and positioning data. However, dynamical changes and spatial features at a location far away from the probe are expected to influence the local dynamics in an averaged way rather than “instantaneously;” the farther away those features are from the probe, the less important their details are likely to be. In this coarse-grained sense, one may integrate a two-dimensional imaging technique with real-time 3D single-particle tracking, with the latter actively guiding the focus of a microscope following the trajectory of a probe particle of interest.

2.7 Outlook

Real-time 3D single-particle tracking spectroscopy has now evolved beyond the initial technical development. Indeed, the challenge ahead is designing new experiments that take advantage of the unique capabilities of this promising approach. Nevertheless, advancements in several fronts will undoubtedly further broaden the problem scope to which it can apply.

There remains a gap between the time and length scales accessible by currently available technology and those of the molecular scale. This gap can be bridged with the development of new optical probes with improved size, brightness, and durability. Several promising directions are already emerging: such as smaller-size semiconducting quantum dots [127], and bright, non-blinking DNA or peptide encapsulated metal clusters [128, 129]. Besides better probes, another way to advance towards molecule-scale movements would be to develop faster piezoelectric stages. Current three-axis piezoelectric stages appear to be limited at a resonance frequency of 1.5 kHz at full swing (50 μm). Stages with a faster response will help to realize the tracking of single molecules, as has been demonstrated in electrophoretic trapping [130]. When integrated with current implementations of real-time single-particle tracking, advances in detector technologies such as two dimensional, CCD-like detectors where each element has the capability of resolving time (time-correlated photon counting) and energy (spectral resolution) with high quantum efficiency will certainly greatly increase
the amount of information obtainable from experiments. Some promising technologies are already being actively developed [131].

Theoretical developments that connect observations with physical parameters [67, 99, 100, 124], and those that optimize existing technologies [113, 132, 133] are just as important as experimental advances. Rigorous theories are crucial to arrive at an interpretation with statistical significance because the mode of nanoscale movement is usually stochastic and the data is most likely inundated with noise [134]; interpretation based on visual inspection neglecting the assumptions underlying any formula can lead to erroneous conclusions, or to seemingly reasonable conclusions that are disconnected from the data.

The past few years have witnessed many breakthroughs in real-time 3D single-particle tracking spectroscopy at a remarkable pace; it is now at a position poised for undertaking many fundamental questions in complex systems.
Chapter 3

Quantitative Characterization of Changes in Dynamical Behavior for Single-Particle Tracking Studies

3.1 Introduction

In 1827, the botanist Robert Brown discovered random movement of pollen grains in solution under a microscope.[135] Brown did not realize that he had started a whole new field of particle tracking. Later on, he observed the same random motion in nonliving particles, leading him to conclude that the observed motions were a natural phenomenon in the microscopic world rather than a reflection of the vitality in the pollen grains. The microscopic origin of the random motion, which was named after him as the Brownian motion, had to wait until 1905 for a final explanation by Einstein,[136] and later by Smoluchowski.[137] One’s visualization ability has since improved significantly with continuous technological innovations (see, for example, the review by Saxton and Jacobson[91]). A modern charge-coupled device (CCD) camera can detect a single fluorescence molecule easily, in contrast to micrometer-sized objects by Brown’s eyes. This type of video imaging experiment has allowed experimentalists to follow the motion of individual particles or molecules projected onto a two-dimensional (2D) imaging plane.[112, 138–145] In these recent experiments, the time-dependent positions (a trajectory) of a single molecule or a single particle is recorded either by a camera[138–141, 143, 144] or by laser scanning.[112, 142] More recently, it has become possible to track individual nanoparticles in three dimensions (3D) by using a laser scanning configuration by either Levi et al.[111] or Cang et al.[107] using a confocal setup that also affords concurrent optical readouts. Tracking experiments can be separated into two general categories. One type of experiment focuses on the object itself; for instance, the study of motor proteins aims at understanding the mechanical motions of the protein associated with enzymatic activities on the molecular level.[138, 141, 143, 145] The other type of experiment aims at understanding the local environment of the probe by observ-
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In general, when the experiments involve a highly heterogeneous environment, biological samples,[147] the properties of the molecule (or probe) being followed are expected to be influenced by its environment. As such, quantitative characterizations of probe dynamics and the changes in them are expected to provide new insight into the microscopic environment and the local fluctuations.

This paper outlines a general procedure to quantitatively detect the changes in the stochastic movement of the probe. The approach is statistically robust, allows accurate extraction of dynamical parameters from a noisy tracking trajectory, and does not require any a priori assumption of a kinetics scheme for analysis. Such model-free approaches (free of presumed kinetic model)[148–151] are especially constructive in arriving at a physical understanding firmly supported by the data;[151, 152] moreover, it allows us to extract more information from an otherwise noisy data set as will be discussed below.

3.2 Background and Formulation of the Problem

In general, the motion of a nanoparticle may include both directional motion and diffusive random walk. Although the approach outlined herein can be generalized to such a compound situation, the present discussion focuses on the extraction of diffusion coefficients and on the detection of their changes along a tracking time trajectory. Here, the diffusion coefficient, $D$, is defined on a time scale that is much longer than the molecular collision time, $t \gg \frac{mD}{k_BT}$, where $m$ is the particle mass, $k_B$ is Boltzmann’s constant, and $T$ is the temperature in Kelvin.[153] On this time scale, the Einstein expression is valid and will serve as the basis for the present development.[154] As an example, one may consider two scenarios caricatured in Figure 3.1, paralleling cases envisioned by Zwanzig.[155] In the first scenario, depicted in Figure 3.1A, the probe remains in a dynamically homogeneous compartment throughout the experiment ($t_0, ..., t_N$), but its dynamical behavior changes at time $t_k$ from $D_1$ to $D_2$ on a time scale shorter than the experimental time resolution. This can be a reflection of a locally uniform transformation in its immediate environment. For instance, in the context of the Langevin equation and the Stokes-Einstein relation, the diffusion coefficient is related to fluctuations in the random force via the fluctuation-dissipation theorem by $D = 2(k_BT)^2 / \int_{-\infty}^{\infty} \langle F(0)F(t) \rangle_0 \, dt$, [156] where $k_B$ is Boltzmann’s constant, $T$ is the temperature in Kelvin, $F(t)$ is the time-dependent random force acting on the probe, and $\langle \cdots \rangle_0$ is the thermal averaging over the equilibrium configuration. Thus, changes either in temperature or in the fluctuating force can result in a change in $D$. In the second scenario, depicted in Figure 3.1B, the probe particle is in a dynamically heterogeneous environment. During the course of a single-particle tracking experiment, the probe may traverse from one dynamical region to another as characterized by different diffusion coefficients. Different dynamical regions are separated by (invisible) boundary lines, assumed to be time-independent of the time scale of the experiment. Thus, the spatial information such as compartmentalization is encoded in the position time trace of the single particle, and can in principle be recovered if the time instance at which the diffusive behavior changes can be determined.
accurately. For example, based in part on the lateral diffusion of decapentaplegic (Dpp) in developing Drosophila cells,[157] Berezhovskii and Weiss have proposed a theoretical model to interpret the lateral diffusion of a particle in a spatially segmented environment.[158] Typically, the treatment of a diffusing particle in a spatially segmented environment is done with a confined or corralled diffusion model. Confined or corralled diffusion treats a diffusing particle as locally confined to some small region in space in which the diffusion coefficient is constant. As the particle approaches the spatial boundary, there is some probability of transition from region 1 to region 2. In the treatment of corralled diffusion, the diffusion coefficient in region 2 is the same as that in region 1. The proposed method therefore can be considered a generalization of the corralled diffusion model where the diffusion coefficients in regions 1 and 2 are not necessarily the same. Quantitative characterizations of single-particle tracking experiments are therefore expected to bring new insight not only into molecular cell biology (since cells are highly compartmentalized) but also into critically testing various microscopic theoretical models.[158–164]

Single-particle tracking can, in principle, provide great insight into local fluctuations because it allows the direct observation of dynamical changes as they occur. Typically, the diffusion coefficient for these particle trajectories is calculated by plotting the mean-square displacement (MSD) versus time delay and fitting the data to a diffusion model. Much of the important theoretical underpinning of this approach and earlier developments in single-particle tracking experiments have been discussed extensively and reviewed by, for example, Qian et al.[99] and Saxton;[91] they are therefore not repeated here. Instead, one is concerned with the deduction of the time-dependent behavior of the diffusion coefficient itself. In contrast to ensemble-averaged experiments, single-molecule experiments rely on time averaging along a measured trajectory. As such, experimental uncertainties become directly coupled to the measurement of a system’s dynamics. This is one of the major challenges in extracting dynamical information from single-molecule experiments. Figure 3.2A shows a simulated trajectory of a particle diffusing in one dimension. The vertical lines indicate the time instances at which its diffusion coefficient changes with the ratio $D_1/D_2 = 1.75$ with the true time-dependent diffusion coefficients shown in the bottom panel. Figure 3.2B depicts the one-step displacement calculated from the trajectory by $\Delta x_i = x_{i+1} - x_i$, with the step size of 0.1 ms. Although the particle’s diffusive behavior changes four times during the measurement, one cannot identify these changes by visual examination. The one-step displacement looks almost flat throughout the entire period as shown in Figure 3.2B. One may attempt to extract the diffusion coefficient using the most commonly used MSD approach.[99] To do so, one bins the trajectory into several segments and, within each segment, calculate $MSD = \langle (x(t + \tau) - x(t))^2 \rangle = 2D\tau$ as a function of the time lag, $\tau$. The subjective choice of bin width impacts the interpretation; a quantitative identification of the dynamical changes by visual inspection is problematic and, as in this case, often not achievable. This issue is illustrated in Figure 3.2C-F, where the MSD-computed diffusion coefficients are displayed as a function of time with bin widths of 5, 25, 75, and 187.5 ms, respectively. The first observation one may make is that binning loses information of the fast dynamics of the system. Oftentimes, it is these high-frequency dynamics probing
Figure 3.1: Drawings illustrating possible scenarios that may cause time-dependent changes in the diffusion coefficient of a single particle. (A) The probe is in a dynamically homogeneous compartment throughout the experiment, but it diffusive behavior changes at time $t_k$. (B) The probe is in a dynamically heterogeneous environment as characterized by the diffusion coefficient. The probe may cross the dividing boundary lines (possibly invisible) during the course of the experiment, resulting in observed changes in the diffusion coefficient. Reprinted with permission from Ref. [67]. Copyright 2006, American Chemical Society.
Figure 3.2: Trajectory for the one-dimensional diffusion of a 250 nm gold nanoparticle exhibiting a time-dependent diffusive behavior was simulated. (a) The particle position as a function of time. (b) One-step displacement as a function of time. (c-f) Diffusion coefficient as a function of time evaluated using the mean-squared displacement method at every 5, 25, 75, and 187.5 ms, respectively. (g) “True” values of the diffusion coefficient as a function of time. There are two diffusive states, 10.3 and 5.89 $\mu$m$^2$/s, intervening at every 75-ms interval. Reprinted with permission from Ref. [67]. Copyright 2006, American Chemical Society.
short-length scale motions that are most revealing regarding local fluctuations. Furthermore, the figure suggests that binning the trajectory at a different time window yields completely different results, which may, in turn, impact on one’s understanding about the true dynamics of the system. The unavoidable measurement uncertainties further complicate one’s ability to extract the underlying dynamics.

In the following section, a systematic approach to analyzing the trajectory of a diffusing particle is described. Characterization of the new method suggests that this method is statistically robust and is able to identify changes in diffusion coefficients accurately, as well as extract them even in the presence of large-amplitude measurement noise. This method is based on the maximum likelihood estimation and draws ideas from statistical test theories. Maximum likelihood estimates exhibit the advantageous property of being asymptotically normally distributed about the true value under most circumstances. It is shown that the true diffusion coefficient can be consistently extracted from the noisy stochastic trajectory with minimal loss of high-frequency information. It is achieved by two steps. First, one identifies the change points in the trajectory and separating the trajectory into different segments. Second, one estimates the diffusion coefficient over each segment. The segmentation of the trajectory using a change-point algorithm segments the trajectory into regions where the value of the estimated parameter is time-independent with certain statistical confidence. Once the segments have been identified, we use the expression for the MLE for the diffusion coefficient to provide the best estimate to the true value even in the presence of measurement noise. The main conclusion is displayed in Figure 3.8, illustrating that the proposed approach does indeed recover the changes in diffusion coefficient from the noise-corrupted trajectory shown in Figure 3.2A.

3.3 Approach

One is mindful of several realistic constraints in devising the proposed approach. The data are usually limited, intrinsically stochastic, and coupled with various experiment noises. Thus, the method should be able to extract as much information as possible. Subjective parameters such as the bin width ought to be avoided. Ideally, one would like the method to be free of presumed kinetic models. As with any physical measurement, an assessment of the uncertainties at given confidence levels should be given. Here, the problems of determining changes in diffusion coefficients and of extracting them from noisy tracking trajectories are mapped onto those of change-point detection and parameter estimation in statistics.

General Considerations Without loss of generality, one considers a particle undergoing free diffusion on a one-dimensional space (1D Brownian motion). Extension to two- or three-dimensional cases is relatively straightforward. The position of the particle is sampled at an experimentally determined time interval, \( \delta \), to give a sequence of \( N + 1 \) positions as a function of time, \( x_i \), where \( x_i \) is measured at chronological times \( t = i\delta \) with \( i = 0, \cdots, N \). Assuming a constant diffusion coefficient, \( D \), the transition probability for the particle to
Figure 3.3: Distribution of the diffusion coefficient, $D$, extracted from simulated single-particle tracking data using the mean-square-displacement approach (dashed lines) evaluated at different time lags ($\tau_{\text{max}}$). The thick solid lines are the distribution using maximum likelihood estimators derived in this paper. Each simulated time trace contains 1024 time points. For both cases, in the absence (A) and in the presence (B) of measurement noise ($\sigma^2/\delta = 2$), 50,000 traces were generated and the extracted diffusion coefficients were accumulated. The true diffusion coefficient was set to 1. Reprinted with permission from Ref. [67]. Copyright 2006, American Chemical Society.
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Figure 3.4: Detection power as a function of the magnitude of changes in diffusion coefficient under the alternative hypothesis in which there is exactly one change point. The solid lines are spline fits as a guide for the eyes. Note that detection power is also dependent on the number of time points included in the analysis. In this simulation, a total of 100,000 traces of 200 Gaussian distributed random numbers were generated and analyzed with the change in diffusion coefficient from $D_1 \rightarrow D_2$ occurring at the 100th point. The critical regions for false-positive detection error used in this analysis are indicated by C1-, where $1 = 0.69$, 0.90, 0.95, and 0.99. Dashed lines indicate 90% and 95% detection powers. Reprinted with permission from Ref. [67]. Copyright 2006, American Chemical Society.
move from $x_i$ to $x_{i+j}$ is, $P(x_{i+j}|x_i) = \frac{1}{\sqrt{4\pi D_j \delta}} \exp\left[-\frac{(x_{i+j} - x_j)^2}{4D_j \delta}\right]$. Therefore, the displacements between juxtaposing position measurements, $\Delta_i \equiv x_i - x_{i-1}$, form a set of normally (Gaussian) distributed random variables $\{\Delta_i\}$ with mean 0 and variance $2D\delta$. That is, one has the probability density of obtaining $\Delta_i$

$$f(\Delta_i; D) = \frac{1}{\sqrt{4\pi D\delta}} \exp\left[-\frac{\Delta_i^2}{4D\delta}\right] \quad (3.1)$$

To include measurement errors, which may arise from mechanical instabilities or from uncertainties in image analysis in video imaging applications, each measurement can be considered as a convolution of the exact position with a Gaussian noise of variance $\sigma_c^2$ that can be characterized independently in control experiments. In a video tracking experiment, for example, the noise also includes the centroid position fitting error.[165] In the unlikely case
Figure 3.6: Standard deviations for the accuracy in identifying the correct time step of a change in dynamical behavior were calculated from 100,000 independent simulations. As the ratio of $D_2/D_1$ approaches 1, the limit of no change, the error in correctly identifying the change increases. The false-positive error rates ($\alpha$) for these curves are, moving inward, 0.31, 0.1, and 0.05, 0.01. Reprinted with permission from Ref. [67]. Copyright 2006, American Chemical Society.

that this is not easily achievable, $\sigma_c^2$ can be deduced by considering it as a fitting parameter. Thus, in the presence of measurement noise, the probability density for obtaining $\Delta_i$ becomes

$$f_c(\Delta_i; D) = \frac{1}{\sqrt{4\pi(D\delta + \sigma_c^2)}} \exp \left[ -\frac{\Delta_i^2}{4(D\delta + \sigma_c^2)} \right]$$

(3.2)

Generalizing this idea to allow for changes in the diffusion coefficient during the course of a measurement, one considers that at some time instance, $t_k$, the diffusive behavior of the particle changes as characterized by a change in its diffusion constant from $D_k$ to $D_{k+1}$. Thus, the time-dependent diffusion constants, $\{D_k\}$, characterize the dynamics of the particle in a coarse-grained manner (piece-wise smooth). The task at hand is then to find the time instances, $\{t_k\}$, at which the diffusion characteristics change and to estimate the corresponding diffusion coefficients, $\{D_k\}$. 
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Figure 3.7: Standard deviations for the accuracy in identifying the correct time step of a change in dynamical behavior were calculated from 100,000 independent simulations. Moving inward, the total number of points in a simulation are 100, 80, 60, and 40 with the change in diffusion coefficient from $D_1 \rightarrow D_2$ occurring at the 50th, 40th, 30th, and 20th point, respectively. As the total number of points in a simulation decreases from 100 to 40 points, the distribution becomes broader and less sharply peaked about the ratio of $D_2/D_1$.

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Maximum Likelihood Estimation of Diffusion Coefficients. The most popular method for analyzing single-particle tracking data has been the mean-square-displacement (MSD) approach.[91, 99] For the one-dimensional case, the diffusion coefficient is calculated using $< (x(\tau + t) - x(t))^2 > = 2D\tau$, where the average $< \cdots >$ is carried out over the entire trajectory and $\tau$ is the time lag between two measurements from which the displacements are to be included in the average. In practice, however, the maximum time lag, $\tau_{\text{max}}$, is often empirically chosen as 1/4 of the total trajectory length[91] because those $\tau > \tau_{\text{max}}$ are not sufficiently sampled to result in exceedingly large statistical noise.[166] A new method that analyzes the spot size on a 2D image has recently been proposed to overcome some of the limitations of the MSD approach.[167] In general, however, an accurate characterization of the diffusion processes is a difficult problem and remains an active area of research in statistics.[168]
Figure 3.8: Number of and estimates for the diffusive states of a 250 nm gold nanoparticle in a temporally heterogeneous environment are quantitatively recovered using a likelihood ratio test to identify the states and the maximum likelihood estimator for diffusion coefficient to evaluate them. The change points were identified at a false-positive rate of 1%. The horizontal error bars give one standard deviation confidence bound for correctly determining the change point. Vertical error bars are the expected standard deviation calculated using the Fisher information. Reprinted with permission from Ref. [67]. Copyright 2006, American Chemical Society.
Here, the maximum likelihood estimators (MLE)\[169\] are derived for the calculation of diffusion coefficients. MLE has many salient statistical properties, making it an ideal choice for extracting physical parameters from single-particle or single-molecule measurements. Parameters calculated using MLE are themselves random variables and are asymptotically unbiased and Gaussian-distributed under most conditions. Furthermore, the use of MLE for diffusion coefficients enters naturally in the determination of diffusion change points within the framework of the log-likelihood ratio test.

Starting with the noise-free case, eq 3.1, the overall likelihood for observing \(N\) displacements, \(\{\Delta_i\}\), is

\[
f(\Delta; D) = \prod_{i=1}^{n} \frac{1}{\sqrt{4\pi D\delta}} \exp \left[ -\frac{\Delta_i^2}{4D\delta} \right]
\]

It follows that the MLE for the diffusion coefficient is one that maximizes the above equation

\[
\hat{D} = \frac{1}{2N\delta} \sum_{i=1}^{n} \Delta_i^2
\] (3.3)

Thus, in the noise-free case, MLE is identical to MSD with \(\tau = \delta\). Equation 3.3 establishes a formal relation between MSD and MLE when there is no measurement noise, the latter being the limiting case of the former. This estimator can be shown to meet the consistency criteria as \(N \to \infty\).[170] The variance associated with this estimation is bound by the inverse Fisher information \(J\) (or Fisher information matrix \(J\) in a multidimensional case)[171–173]

\[
\text{var}(\hat{D}) \geq J^{-1} = (NJ_1)^{-1} = \frac{2D^2}{N}
\] (3.4)

where \(J_1\) is the Fisher information contained in one measurement of \(\Delta_i\). The equality is true when an unbiased estimator is used, as in the present case using MLE. The last term also reaffirms that information is additive. In addition, the relative error can be seen to scale as inverse square-root of \(N; \sqrt{\text{var}(\hat{D})}/\hat{D} = \sqrt{2/N}\).

Similarly, in the presence of additive Gaussian noise, the overall likelihood is

\[
f_c(\Delta; D) = \prod_{i=1}^{n} \frac{1}{\sqrt{4\pi(D\delta + \sigma_c^2)}} \exp \left[ -\frac{\Delta_i^2}{4(D\delta + \sigma_c^2)} \right]
\]

which gives the MLE expression for the diffusion coefficient

\[
\hat{D}_c = \frac{1}{2N\delta} \sum_{i=1}^{n} \Delta_i^2 - \frac{\sigma_c^2}{\delta}
\] (3.5)

This analysis suggests that measurement noise introduces a systematic bias \((\sigma_c^2/\delta)\) that is dependent on the magnitude of the noise as well as on the sampling time scale. If the noise term is not included in the MLE analysis, for example, then the apparent diffusion
coefficient contains a non-vanishing positive bias, \( D_{\text{app}} = \sum_{i=1}^{N} \Delta_{i}^{2} / (2N\delta) = (D_{c}) + \sigma_{c}^{2} / \delta \equiv D_{\text{true}} + \sigma_{c}^{2} / \delta \). The distribution of the MLE-extracted diffusion coefficient is also broadened by the measurement noise, as indicated by the expected variance lower bound

\[
\text{var}(\hat{D}_{c}) \geq J_{c}^{-1} = (NJ_{1c})^{-1} = \frac{2(\sigma_{c}^{2} + \delta D)}{N\delta^{2}}
\]  

(3.6)

where \( J_{c} \) and \( J_{1c} \) are the total and the single-measurement Fisher information, respectively. Thus, the expected relative error in the presence of measurement noise scales as 
\[ \sqrt{\text{var}(\hat{D}_{c}) / (\hat{D}_{c})} = \sqrt{2/N(1 + \sigma_{c}^{2} / \delta \hat{D}_{c})} \]

The above analysis also suggests that in order to make reasonable measurement in the presence of noise one either samples at a slower rate (longer \( \delta \), an experimentally controllable parameter) or uses more samples (larger \( N \)) in the evaluation of diffusion coefficient.

To assess the performance of the MLE approach, we compared the distribution of the MLE-estimated diffusion coefficient with that of the MSD. Following Saxton,\(^{[91]}\) 1024 time points were simulated using \( D_{\text{true}} = 1 \) and \( \delta = 1 \). The distributions of MSD-estimated \( D_{\text{MSD}} \) at various \( \tau_{\text{max}} \) were accumulated over 50 000 independent simulations. As shown in Figure 3.3A, it is evident that the spread of extracted diffusion coefficient using the MSD approach narrows as \( \tau_{\text{max}} \) becomes smaller. One might surmise that when \( \tau_{\text{max}} = \delta \), the “best” estimate can be obtained. This conjecture is indeed true because earlier this section has formally established that MLE is a limiting case in the MSD-type approach, thereby providing a theoretical justification for analyses that use short \( \tau_{\text{max}} \) in MSD treatments. In addition to its optimality, the MLE approach gives a consistent and narrow distribution, the width of which is determined by the Fisher information. To characterize how measurement noise affects extraction of diffusion coefficients using these two different approaches, a Gaussian noise (\( \sigma_{c}^{2} / \delta D = 2 \)) was added to the time traces used to generate Figure 3.3A, and the distribution of \( D \) extracted at various \( \tau_{\text{max}} \) were accumulated. As discussed earlier, measurement noise tends to introduce a positive bias in the extracted diffusion coefficients if it is not properly accounted for. This is seen clearly in the case when \( D \)s are extracted using the MSD approach as shown in Figure 3.3B. In contrast, the MLE approach yields a consistently unbiased distribution, the width of which can be explained quantitatively by eq 3.6.

Having illustrated that MLE is an efficient and statistically robust approach to extracting diffusion coefficients from single-particle tracking data, one next discusses the issue of following the changes in the diffusion coefficient along a single-particle tracking time trace. In cases when the diffusion coefficient changes on a time scale that is slower than the experimental sampling time, the MLE expressions (eqs 3.3 and 3.5) and the Fisher information (eqs 3.4 and 3.6) can be used to construct a maximum-information procedure to obtain the evolution of the diffusion coefficient as a function of time,\(^{[148]}\) \( \hat{D}(t) \) (a coarse-grained trajectory), from which the distribution of can also be recovered quantitatively using information-theoretical approaches.\(^{[151]}\) The next section outlines how to correct the measured diffusion coefficient for errors due to the finite exposure time of cameras used in video tracking experiments.
Effect of Camera Exposure Time or Frame Averaging on the Apparent Diffusion Coefficient. One of the limitations to the accurate determination of changes in diffusive behavior in single-particle video tracking experiments may be due to the exposure time of the camera. As the exposure time increases, the measured diffusion coefficient will decrease. Ritchie et al. [174] have shown that the observed dynamics of a diffusing particle undergoing confined or hop diffusion are dependent on the choice of exposure time in a video tracking experiment. An analytical expression that relates the exposure time to the measured apparent diffusion coefficient is presented below.

Let $\delta$ be some unit time over which the positions $x_i$ can be defined by video microscopy. For example, at $t_1$ the particle is at position $x_1$, and $t_2 = t_1 + \delta$ at $x_2$, and so forth. For a Brownian particle, the positions are related by $x_{i+1} = x_i + \Delta_i$, where the displacements $\{\Delta_i\}$ are Gaussian with zero mean and variance $2D\delta$. For clarity, the detection noise is neglected and the case is limited to one dimension in this discussion. The derivation can be extended to two or three dimensions easily. Let each camera frame be taken at an interval of $m\delta$ with an exposure time of $q\delta$ ($m \geq q > 0$). Therefore, the apparent position that one obtains at each frame is the average of $q$ positions over the exposure time. For example, the mean positions for frames 1 and 2 are, respectively

$$\bar{x}_q^{(1)} = x_1 + \sum_{j=1}^{q-1} \frac{q-j}{q} \Delta_j$$

and

$$\bar{x}_q^{(2)} = \bar{x}_q^{(1)} + \sum_{k=1}^{q-1} \frac{q-k}{q} \Delta_k$$

$$= x_1 + \sum_{j=1}^{m} \frac{q-j}{q} \Delta_j + \sum_{k=1}^{q-1} \frac{q-k}{q} \Delta_k$$

Therefore, the apparent displacement between frames 1 and 2 is

$$\bar{x}_q^{(2)} - \bar{x}_q^{(1)} = \sum_{j=1}^{q} \frac{j}{q} \Delta_j + \sum_{i=q+1}^{m} \Delta_i + \sum_{k=1}^{q-1} \frac{q-k}{q} \Delta_k$$

The apparent diffusion coefficient, $D_{app}$, is the mean of the maximum likelihood estimates of the diffusion coefficient from $N$ frames. Let $E \cdots$ denote the expectation value. Then $D_{app}$ can be evaluated by

$$D_{app} = E\{D_{MLE}\} = \frac{1}{2(N-1)(m\delta)} \sum_{n=1}^{N-1} E\{(\bar{x}_q^{(2)} - \bar{x}_q^{(1)})^2\} = \frac{D}{m} \left[ m + \frac{1}{3q} - \frac{q}{3m} \right]$$

Thus, one has $D_{app}/D = 1 + 1/3mq - q/3m \leq 1$. When $q = 1$, $D_{app}$ converges to $D$ as expected. At the other extreme when exposure time equals frame rate$^{-1}$, or $q = m$, one has
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$D_{app}/D = 2/3 + 1/3m^2$. Therefore, the limiting case for the apparent diffusion coefficient when taking into account exposure time is $D_{app}/D = 2/3$. If $q/3m \gg 1/3mq$, a condition that can be easily satisfied in typical experiments, then the ratio reduces to

$$\frac{D_{app}}{D} \approx 1 - \frac{1}{3} \left( \frac{T_{\text{exposure}}}{T_{\text{frame}}} \right)$$

where the last term denotes the ratio of exposure time over the frame interval. The above equation provides a convenient formula to rescale the measured apparent diffusion coefficient to approximate the true value. An expression equivalent to the latter approximation has been reached by Savin and Doyle,[175] albeit via a different derivation. One notes that the same formula can be applied to cases where several frames are averaged in order to achieve a better signal-to-noise ratio. In addition, the derivation outlined here can be readily extended to expressions that are suitable for two- or three-dimension cases. The next section presents a statistically robust approach that deals with cases when the diffusion coefficient changes on a time scale faster than the measurement time, $\delta$.

Detection of Changes in the Diffusion Coefficient

The form of the distribution for observing a position change suggests that finding change points in diffusive behavior reduces to the problem of finding change points in the variance of a Gaussian distribution. Following Horváth, identifying changes in $D$ amounts to testing the alternative hypothesis $H_A$, where there is a sudden change in $D$ at time index $i$, against the null hypothesis $H_0$, where the trajectory forms a random walk with an uniform diffusion coefficient:[176]

$$H_A : D(t_1) = D(t_2) = ... = D(t_i) = D_1$$
$$\neq D_2 = D(t_{i+1}) = ... = D(t_N)$$
$$H_0 : D(t_1) = D(t_2) = ... = D(t_N) = D_0 \quad (3.7)$$

To test hypothesis $H_A$ against the null hypothesis, $H_0$, one calculates twice the log likelihood ratio (llr) of the two hypotheses. When there is no measurement noise, this ratio is a function of index $k$ and is given by

$$2 \times llr(k) = 2 \ln \left[ \frac{f(\{\Delta_{i=1...k}\}|\hat{D}_1) f(\{\Delta_{i=k+1...N}\}|\hat{D}_2))}{f(\{\Delta_{i=1...N}\}|\hat{D}_0)} \right]$$
$$= N \ln \left[ \hat{D}_0 \right] - k \ln \left[ \hat{D}_1 \right] - (N-k) \ln \left[ \hat{D}_2 \right] \quad (3.8)$$

where $\hat{D}_1 = (2k\delta)^{-1} \sum_{i=1}^{k} \Delta_i$, $\hat{D}_2 = [2(N-k)\delta]^{-1} \sum_{i=k+1}^{N} \Delta_i^2$, and $\hat{D}_0 = (2N\delta)^{-1} \sum_{i=1}^{N} \Delta_i^2$ are the MLE values for the diffusion coefficients. This ratio is a measure of how likely that there is a sudden change in the diffusion coefficient at index $k$. The most likely location for the change to occur is the index that maximizes the log-likelihood ratio, $Z_N^{1/2} = \max_{1 \leq k \leq N} \{2llr(k)\}$. 
The probability for a diffusion transition to occur is \( P(Z_{1/2}^N \geq C_{1-\alpha}) = 1 - \alpha \), where \( \alpha \) is the probability of having a false-positive identification of a change point (Type-I error), and \( C_{1-\alpha} \) is the critical region associated with this \((1 - \alpha)\) confidence level. In other words, if \( Z_{1/2}^N \geq C_{1-\alpha} \), then the diffusive behavior of the particle is considered to change with a probability \( \alpha \) that it is a false identification. However, the particle is considered to be undergoing Brownian motion with a uniform diffusion coefficient if \( Z_{1/2}^N < C_{1-\alpha} \). Thus, the statistical test used to determine the location at which the diffusion coefficient changes is

\[
\text{Test}: \begin{cases} \frac{Z_{1/2}^N}{N} \geq C_{1-\alpha}, & \text{there is a changepoint at index} \\ \frac{Z_{1/2}^N}{N} \leq C_{1-\alpha}, & \text{there is no change point contained in the data set.} \end{cases}
\]

Generally, the most challenging part in formulating a rigorous statistical test is the evaluation of the critical region. Fortunately, for testing changes in variance of a Gaussian distribution, the asymptotic critical values for Type I error rates have been derived in closed forms by Horváth.[177] For completeness, they are reproduced here

\[
C_{1-\alpha} = \frac{b(\ln(N)) - \ln[-(1/2)\ln(\alpha)]}{a(\ln(N))} \quad (3.9)
\]

where \( a(u) = \sqrt{2\ln(u)} \) and \( b(u) = 2\ln[u] + \ln[\ln(u)] \). The critical values in eq 3.9 are the asymptotic critical values, valid only in the limit \( N \to \infty \). Gombay and Horváth[176] showed that eq 3.9 overestimates the critical region and derived an improved approximation to the true critical region

\[
\frac{C_{1-\alpha}^2 \exp\left(-\frac{C_{1-\alpha}^2}{2}\right)}{2} \left\{ T - \frac{2}{C_{1-\alpha}^2} \right\} + 4 \frac{C_{1-\alpha}^2}{C_{1-\alpha}^2} = 1 - \alpha \quad (3.10)
\]

where \( T = \log((1 - h)^2/h^2) \) and \( h = \log(n)^{3/2}/n \). The critical region in eq 3.10 can be easily solved numerically. In one dimension, the method presented above is also applicable to the case of a nonzero mean in the probability density, corresponding to directed motion.[178]

Using the critical values (eq 3.10) and the expression for the log likelihood ratio (eq 3.10) one can construct a recursive binary segmentation algorithm[149] to quantitatively recover all the time points, within given confidence interval and error rate, that signals a change in the diffusive behavior along the position time trace. The same test can also be applied to an actual experimental data trace without modification. In the presence of noise, this algorithm detects the changes in the effective variance, \((2D_1\delta + 2\sigma_c^2) \to (2D_2\delta + 2\sigma_c^2)\), as the particle traverses from region 1 to region 2. The performance of the algorithm, however, degrades rapidly as the magnitude of the noise relative to \( D\delta \) becomes greater. Some performance issues are discussed below.

Power of Detection. An important parameter characterizing a statistical test is the power of the test. For the present case, the power of diffusion change-point detection is the probability of detecting a change in diffusion coefficient when it is known there is a
change along the tracking trajectory. Like the false-positive critical region (eq 3.9), the power of detection is dependent on the number of data points included in the test; the more data points included, the more powerful the test becomes. In addition, the power is also determined by the size of the change, characterized by the ratio $D_2/D_1$. Because a close-form expression for this test is not yet available, one characterizes the power of this test using computer simulations.

The following parameters were used for the simulations used in the remainder of this section. A random-walk trajectory was simulated by drawing 100 Gaussian distributed random numbers with a variance of $2D_1\delta$, concatenated by an additional 100 Gaussian numbers but with a variance of $2D_2\delta$. The exact numeric values for $D$ and $\delta$ are unimportant for these purposes because the relevant parameter here is the ratio $D_2/D_1$. This 200-point trace was then analyzed using the diffusion change-point algorithm with confidence intervals $C_{0.09}$, $C_{0.90}$, $C_{0.95}$, and $C_{0.99}$. The results from 50 000 traces were accumulated and further analyzed.

The results for the power of detection are summarized in Figure 3.4. For example, to achieve 90% detection power, the minimum ($D_2/D_1$) ratios are 2.25 for $\alpha = 0.31$, 2.76 for $\alpha = 0.1$, 2.97 for $\alpha = 0.05$, and 3.65 for $\alpha = 0.01$. For a finite number of data points, it has been shown that the asymptotic expression overestimates the true critical region,$[177]$ to cause a lower-than-expected detection power. The critical region used in this implementation also overestimates the critical region by 16.7% for $\alpha = 0.31$, 6.4% for $\alpha = 0.1$, 3.5% for $\alpha = 0.05$, and 0.7% for $\alpha = 0.01$. However, the detection power is independent of the manner the diffusion behavior changes, but only dependent on their relative size; that is, power($D_2/D_1$) = power($D_2/D_1$). For experiments that track individual fluorescent molecules, where the trajectories can contain less than 100 points or camera frames in length, the proposed method can still detect changes in diffusive behavior. However, for a fixed value of $D_2/D_1$, the detection power will decrease as the number of points in a trajectory decreases. For example, in Figure 3.5, the detection powers, at a ratio of $D_2/D_1 = 10$, are 99.9, 99.5, 95.9, and 75.3% for trajectory lengths of 100, 80, 60, and 40 points. In comparison, for a trajectory 200 points long the power of detection reaches 100 percent at a ratio of $D_2/D_1 = 10$.

Confidence Interval. Another important parameter to be characterized is the accuracy to which the location of diffusion change is determined. An exact formulation for the confidence levels of the detected diffusion change points does not appear to be available.$[179]$ Therefore, one turns to computer simulations to gain an assessment of the accuracy for the method. In these simulations, the standard deviations of the detected change points with respect to the true location (the 100th time point) are accumulated as a function of the ratio ($D_2/D_1$). The results are summarized in Figure 3.6. It was found that, for example, the location at which diffusion behavior changes can be isolated accurately with an uncertainty of 20 time points when $D_2/D_1 = 2$. The confidence limits for the index of the detected change point will also decrease as the length of the trajectory decreases. Simulating single fluorescent molecule tracking experiments, as shown in Figure 3.7, the standard deviations at a ratio of $D_2/D_1 = 1$ are 24.8, 21.7, 17.0, and 10.2 for trajectory lengths of 100, 80, 60, and 40 points. This type of characterization is also applicable to realistic data set that contains measurement
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noise because the relevant parameter that governs the accuracy of the detected diffusion change location is the ratio \( (2D_1\delta + 2\sigma_c^2)/(2D_2\delta + 2\sigma_c^2) \). Therefore, in practical applications, one builds a look-up table for the standard deviation in change-point location that is a function of (1) the number of data points before the detected change point, (2) the number of data points after the detected change point, and (3) the relative size of the change, \( (2D_1\delta + 2\sigma_c^2)/(2D_2\delta + 2\sigma_c^2) \). This look-up table thus allows an experimentalist to assign confidence levels to the detected change-point locations. For example, a 1500 point look-up table was generated to estimate the standard deviation in locating the change points as displayed in Figure 3.8. The next example illustrates that new information that is otherwise hidden in noisy trajectories can be extracted using the above-outlined procedure.

3.4 Example

To mimic a real experimental situation, Gaussian white noise with a standard deviation of 20 nm was added to the trajectory shown in Figure 3.2A. Video tracking can achieve an accuracy of about 1 nm in two dimensions; therefore, the 20-nm uncertainty used in this characterization is a conservative estimate. This gives a \( \sigma_c^2/D\delta \) about 0.7. The corrupted position time-trace and the corresponding one-step displacement are shown in Figure 3.8A and B, respectively. As in the case of Figure 3.2, it is next to impossible to visually identify those changes. When applying the change-point algorithm to the trajectory, one can readily identify five different segments, shown as the solid line in Figure 3.8C, overlaid with the true time-dependent diffusion coefficient plotted as a solid dot line. For this trajectory, the true number of segments was identified. Moreover, the only user-supplied parameter required to apply the change-point algorithm is the desired confidence level (in this case, a 1% false-positive error rate was used) for observing a change. The vertical error bars represent the expected standard deviation for the estimated diffusion coefficient as given by Fisher information (eq 3.6). The horizontal error bars are estimates for the uncertainties in the location of the diffusion change points. They are computed from a look-up table constructed using computations like Figure 3.5. The fact that not all of the one-standard-deviation error bars contain the true values in either the diffusion coefficient or the change-point locate is not unexpected because, for normally distributed variables, one standard deviation includes a 69% confidence level. Therefore, the proposed approach can indeed quantitatively recover the underlying dynamics even in the presence of measurement noise.

3.5 Conclusions

Changes in the diffusion coefficient during a single-particle tracking experiment are usually indicative of variations in some physical parameters, such as the local environments or the probe’s intrinsic states. Yet, a quantitative approach to isolating the changes along an experimental time trace appears lacking. This article contributes an effective and statisti-
cally robust procedure for the extraction of diffusion coefficients and the changes in them from single-particle or single-molecule tracking experiments. It is shown that diffusion coefficients determined using the commonly practiced binning procedure gravely depends on the bin size. Therefore, it is unable to provide a consistent and objective view of the microscopic processes. Furthermore, experimental noise is found to add a positive bias on the estimated diffusion coefficient if not treated properly. To this end, maximum likelihood estimators (MLE) are derived. It is shown that the MLE in the noise-free scenario is a limiting case of mean-squared displacement (MSD), thus formally relating these two approaches. The MSD method, however, fails in the presence of noise while the MLE consistently gives narrow and well-defined distribution given by the Fisher information. A likelihood ratio test, based on the previously established framework in determining fluorescence intensity changes, is applied to locate the changes in the diffusion coefficients. Using the present approach, it is now possible to quantitatively extract dynamical information from single-particle tracking data with proper confidence intervals. Characterization of the procedure shows that it performs well even in the presence of high experimental noise.
Chapter 4

Spectral Anisotropy of Gold Nanoparticles

4.1 Abstract

Metallic nanoparticles synthesized by solution-phase chemistry usually exhibit various polygonal morphologies. The shape is known to have a great impact on a nanoparticles optical properties, for instance, the surface plasmon resonance frequency. It remains unclear, however, whether the scattering spectrum of nanoparticles is generally anisotropic in the far field as a result. This simple question turns out to be extremely challenging to address because of the particle-to-particle shape inhomogeneity in a bulk sample, and the high sensitivity of surface plasmon resonance to local environments. We report the observation of scattering angle-dependent spectra using a newly developed single-particle tracking spectroscopy (SPS). Furthermore, we show that SPS has provided a way to directly visualize the rotational random walk of individual gold nanoparticles in water for the rst time.

4.2 Introduction

Gold and silver nanoparticles exhibit bright colors due to surface plasmon resonance, a coupled oscillation of electromagnetic waves and the free electrons in the particles [53]. This property has been exploited for many applications [180–184]. The surface plasmon resonance is sensitive to several parameters, including the density of the electrons, the dielectric constants of the nanoparticle and the medium, and the shape of the nanoparticles [185–187]. There have been much efforts devoted to nding robust methods to tune these parameters to tailor nanoparticles for specic applications, [188–190] the success of which relies on a fundamental understanding of how these parameters affect the surface plasmon resonance [191]. Among them, the effects of the shape are the most complicated, and also least understood. Since nanoparticles prepared by solution-phase chemistry tend to exhibit a variety of shapes rather than be perfectly spherical (cf. Figure 4.2), one of the intuitive questions is there-
fore: Will the scattering spectrum from a single nanoparticle generally be anisotropic as well? That is, will the color of a nanoparticle illuminated by white light look differently when being observed from different angles [cf. Figure 4.1a]? Surprisingly, this has not been confirmed experimentally, reflecting the great challenges in studying nanoparticles. First, the nanoparticles are highly inhomogeneous. A bulk sample in ensemble-level experiments contains randomly oriented nanoparticles of various shapes; thus, even if every nanoparticle scatters anisotropically, the bulk sample will still appear spectrally isotropic. Second, the signal from a single nanoparticle is weak. Single nanoparticle-level experiments have to efficiently suppress noise from background. There are recent attempts to examine the angle-dependent spectral distribution in the near-field by scanning an optical probe around a single immobilized nanoparticle and taking snapshot spectra at different angles. [192] Spectral anisotropy has been detected from nanorods [192]. The sensitivity of the experiment is low, however. For most nanoparticles that are nonspherical in shape but less elongated, the experiment showed no direction dependence in the scattering spectrum. In addition, the substrate necessary for immobilization is known to introduce anisotropy in the nanoparticles immediate dielectric environment, [193, 194] an effect that is difficult to eliminate. The emerging single-particle tracking spectroscopy has provided a surprisingly easy way to detect the color anisotropy. The single-particle tracking spectroscopy has been independently developed recently by several groups as a way to track the movements of individual nanoparticles by managing the nanoscale Brownian motion through a feedback control [107, 112, 195, 196]. The nanoparticle thus appears as if it is immobilized at the focus of a microscope. This, in turn, allows further investigation of the moving nanoparticle by other spectroscopies. By letting a nanoparticle move unrestrictedly in water rather than fixing it on a substrate guarantees that the nanoparticles dielectric environment is uniform. By tracking, the scattering spectrum is simultaneously recorded by a detector with a detection angle defined by the numerical aperture (NA) of the microscope objective. Due to random collisions with water molecules, the nanoparticle reorients stochastically. In this way, the tracking spectrometer is essentially statistically sampling the entire solid angle of the nanoparticle (cf. Figures 4.1 b-d). If the light scattering is isotropic, the observed spectrum should not change over time. Our experiments showed that the color of the light scattered from individual nanoparticles are, in fact, fluctuating.

4.3 Spectral and Polarization Fluctuations

Figure 4.1e shows the schematic diagram of the instrument. The experimental principles and concepts of three dimensional (3D) single-particle tracking have been described in detail previously. 20 Brie, a water solution containing gold nanoparticles (diameter=80250 nm; Ted Pella) was sandwiched between two cover glasses separated by about 200 \( \mu m \). The sample was then placed on a 3D translation stage, and illuminated by a tungsten lamp through a darkeld condenser (Olympus, NA=1.2). The scattered light was collected by a microscope objective (Leica, NA=0.7). A home-built confocal 3D tracking device monitored
Figure 4.1: Single-particle tracking spectroscopy: (a) Illustration of angle-dependent plasmon scattering from a nearly spherical metallic nanoparticle when illuminated by a white light source. [(b)(d)] Experimental design concept for detecting spectral anisotropy by time-dependent spectroscopy on a freely diffusing gold nanoparticle. As the nanoparticle rotates stochastically in water, its angle-dependent scattering spectrum is sampled statistically by the spectrometer. (e) A schematic of the single-particle tracking spectrometer. A portion of the collected scattering light is directed into a spectrometer. The spectrometer can be configured to measure either the spectral contrast or the polarization contrast as shown in panels (f) and (g), respectively. (f) After a polarizer, the spectral contrast is measured by a dichromatic beam splitter that sorts the light into the red (R) and the blue (B) channels, and detected by corresponding avalanche photo-diodes (APDs). (g) To measure the polarization contrast, a polarization beam splitter categorizes the light into the mutually orthogonal vertical (V) and horizontal (H) components, and detected by corresponding APDs. Reprinted with permission from Ref. [69]. Copyright 2008, American Institute of Physics.
the displacement of the nanoparticle of interest and commanded the stage to counter any translational Brownian movements by steering the cover glasses to keep the nanoparticle at the focus. A beam splitter deflected part of the scattered light from the tracked particle into a spectrometer. A charge coupled device camera (Cascade 512B, Roper Scientific/Photometrics) mounted after a monochromator (Spectra Pro 150, Acton Research) was used to record the spectrum. Figure 4.2 shows the scattering spectrum of a 100 nm gold nanoparticle and demonstrates the capability of recording spectrum of a freely moving nanoparticle for the first time. If a nanoparticle scatters anisotropically, the observed spectrum will vary as the nanoparticle rotates in water (cf. Figs. 4.1b-d). To detect such spectral variations requires sampling the spectrum faster than the nanoparticles rotation. Unfortunately, the light scattered from a single nanoparticle is weak such that it takes a long integration to acquire enough photons at each wavelength for constructing a spectrum. Yet, to determine whether the spectrum fluctuates, it is neither efficient nor necessary to record the entire spectrum.
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Figure 4.3: Spectra anisotropy and color bunching: (a) Coordinate system of a spheroidal nanoparticle freely rotating in water with respect to the laboratory frame ($\hat{X} - \hat{Y} - \hat{Z}$). The thick arrow is a vector depicting the orientation of the spectrally anisotropic nanoparticle. Using The RayleighGans electrostatic approximation for spheroids (Refs. [197, 198]), the nanoparticle is assumed to scatter red light more efficiently when the nanoparticle is at an orientation with the arrow aligned with the $\hat{X}$ axis (Ref. [124]). (b) A 3D representation of detected color contrast $\chi_c(\theta, \phi)$ [see main text Equation 4.1 for definition] for the model in (a) at different orientations defined by the azimuth ($\theta$) and zenith ($\phi$), where realistic experimental parameters are used. As the spectrally anisotropic nanoparticle undergoes rotational random walk, its orientation, represented by the white spots in the unit sphere, will also change to result in corresponding changes in the detected color contrast. Note that it is the relative changes in color contrast that gives evidence for spectrally anisotropic scattering, not the absolute value. (c) A cartoon illustrating how the scattering spectrum (recorded as color contrast) changes as the spectrally anisotropic nanoparticle dynamically reorients itself over time. (d) A typical color contrast time trace, $\chi_c(t)$, from a 100nm gold nanoparticle freely moves in water. To avoid congestion in visualization, the trajectory (originally recorded with 10$\mu$s time resolution) is binned at 100ms, and only one out of every five data points are shown. [(e)(h)] Scattered plots of $\chi_c(t + \delta t)$ against $\chi_c(t)$ at various time delays $\delta t$ to visualize color correlation in the trajectory shown in (a). A spectrally anisotropic nanoparticle scattering with color $\chi_c(t)$ at time $t$ is likely to scatter the same color at time $t + \delta t$ when $\delta t$ is small compared to its reorientation timescale. This is indicated by the diagonally elongated pattern at short-time delays (red dashed line; eye guide). The correlation is quantified by the correlation coefficient $r$. The probability of observing color bunching diminishes and eventually disappears at longer time delays to result in an uncorrelated scatter pattern in the plot with the correlation coefficient approaching 0. Reprinted with permission from Ref. [69]. Copyright 2008, American Institute of Physics.
Figure 4.4: Spectral contrast autocorrelation: Autocorrelation of spectral contrast as a function of time $C_{\chi_c\chi_c}(t)$. The green trace is from an 80nm gold nanoparticle in 80% glycerol/water (w/w) mixture and is overlaid with a fit to a single exponential decay of time constant 11.5ms (black curve). The pink trace is from a 500nm silicate micro-sphere suspended in water, and the dark brown curve from an immobilized 100nm gold nanoparticle. The trajectories used to calculate the correlation functions are 13, 1.28, and 5.18s for the pink, green, and brown curves, respectively. Reprinted with permission from Ref. [69]. Copyright 2008, American Institute of Physics.

Instead, we propose a color binning scheme defined by a spectral contrast function,

$$
\chi_c(t) = \frac{\int_{\lambda_c}^{\infty} F(\lambda, t) d\lambda - \int_{0}^{\lambda_c} F(\lambda, t) d\lambda}{\int_{\lambda_c}^{\infty} F(\lambda, t) d\lambda + \int_{0}^{\lambda_c} F(\lambda, t) d\lambda} = \frac{I_R(t) - I_B(t)}{I_R(t) + I_B(t)}
$$

(4.1)

where $F(\lambda, t)$ is the scattering spectrum of the particle at time $t$ is the cutoff wavelength that defines the contrast, and $I_R \equiv \int_{\lambda_c}^{\infty} F(\lambda, t) d\lambda$ and $I_B \equiv \int_{0}^{\lambda_c} F(\lambda, t) d\lambda$ are the red and blue fraction of the spectrum, respectively. $\chi_c(t)$ can be easily measured using a dichromatic mirror with a transition band edge at $\lambda_c$ and a pair of detectors integrating every 10 $\mu$s (Fig. 4.1f). Here, a standard Chroma 560DCLP filter was used. For a given experimental configuration, $\chi_c(t)$ is greater for a nanoparticle scattering a spectrum that contains more long-wavelength components, and vice versa (Figs. 4.3a-c). Thus, the spectral contrast $\chi_c(t)$ can be a sensitive reporter to detect any variations of $F(\lambda, t)$. A typical $\chi_c(t)$ time trace from a 100 nm nanoparticle in water is shown in Figure 4.3d. However, it is still not apparent from the very noisy trace if the scattering spectrum varies with time. Indeed, objectively extracting information from noise-limited experimental data is one of the challenges in single-
Figure 4.5: Test of the DSE relation: (a) Correlation between color contrast fluctuation-correlation time and hydrodynamic radius of gold nanoparticles in water on a log-log plot. The four samples studied in this experiment were gold nanoparticles suspended in water with nominal sizes 80nm (green), 100nm (red), 150nm (blue), and 200nm (black). The hydrodynamic radius of individual nanoparticles was measured from translational Brownian motion. The dashed black line is the DSE rotational diffusion model with $l = 2$ at $T = 295K$. The light turquoise-shaded area bounded between two red dashed lines corresponds to 15% deviation from the DSE line. Inset: Typical color contrast fluctuation-correlation decay curves for 80, 100, and 150nm nanoparticles. The black dashed lines are fits to single exponential with fitted time constants $0.096 \pm 0.006$, $0.14 \pm 0.02$, and $0.40 \pm 0.04$ms for the 80, 100, and 150nm nanoparticles, respectively. (b) Correlation between polarization contrast fluctuation-correlation time and hydrodynamic radius of gold nanoparticles in water on a log-log plot with the same color coding as in (a). Inset: A representative 3D trajectory of a 100nm nanoparticle (14s long). The position trajectory was recorded at 10kHz. To avoid congestion in visualization, here the nanoparticles 3D position is plotted every 10ms. Reprinted with permission from Ref. [69]. Copyright 2008, American Institute of Physics.

molecule spectroscopy [199, 200]. In the present case, the noise primarily comes from the counting noise reflecting the quantal nature of photons. Ideally, the spectral contrast $\chi_c(t)$ should be a fraction number between 1 and 1, defined by the scattering spectrum $F(\lambda, t)$. This is not the case in practice, however. Consider the case of detecting a photon in an experiment. Depending on the color of the photon, it has to either pass the dichromatic mirror (Figure 4.1f to reach R, or be reflected to reach B, yielding two discretized values for $\chi(t)$, 1 and 1, respectively, with a probability determined by $F(\lambda, t)$. In principle, the spectral contrast could be recovered by averaging over many such single-photon detection events, where the number of photons determines the signal-to-noise ratio. In the experiments, however, the typical photon counting rate is 1001000 counts/s. Consequently, there are only few photons in the 10 $\mu$s acquisition period; therefore, the photon-counting noise dominates the fluctuations seen in the trajectory. To discriminate the fluctuation of $F(\lambda, t)$ from the
photon counting noise, we investigate the correlation of the spectral contrast at two different times $\chi(t)$ and $\chi(t + \delta t)$. If the apparent changes in the spectral contrast are predominantly due to photon counting, $\chi(t + \delta t)$ will be independent of $\chi(t)$ because the photon-counting noise is not correlated in time. On the other hand, if the spectral fluctuates is caused by the reorientation of the particle, the fluctuation will exhibit “color bunching” at short times: a red photon should be statistically more likely followed by another red photon, and a blue photon followed by another blue photon (Figure 4.3c). Since the reorientation is stochastic, eventually, the orientation of the particle will be randomized and so will be the spectral contrast correlation. The analysis is summarized in Figures 4.3e-h, showing the scattered plots of $(\chi_c(t), \chi_c(t + \delta t))$ at various delay time $\delta t$. It is apparent that at short-time delays, $\chi_c(t)$ and $\chi_c(t + \delta t)$ are correlated so that the plot scatters around the diagonal $\chi_c(t) = \chi_c(t + \delta t)$ line. At longer delays, the dots distribute randomly around the mean value $\langle \chi_c(t) \rangle$. The correlation can be quantified by the coefficient of correlation,

$$r = \frac{\sum_i \chi_c(t_i + \delta t)}{\sqrt{\left( \sum_i \chi_c(t)^2 \right) \left( \sum_i \chi_c(t + \delta t)^2 \right)}}$$

The positive correlation at short delays (e.g., $r=0.66$ at $\delta t=0.1$ ms) and the loss of correlation at long delays (e.g., $r = 0.01$ at $\delta t=10$ ms) in Figures 4.3e-h therefore serve as direct evidence for the anisotropic scattering spectrum. The degradation in color correlation provides a means to measure the reorientation relaxation of the gold nanoparticles. In particular, it has not been possible to follow how a single gold nanoparticle rotates in solution so far. In addition to fundamental interests in reorientation relaxation such as the effects of boundary conditions and shapes, the ability to do so quantitatively also has important implications. For example, just as molecular rotation is an integral part of a chemical reaction, the manner in which a nanostructure rotates in solution plays a central role in understanding of such processes as the assemblage of higher hierarchical structures from nanoscale building blocks and biological activities in general [70]. To quantify the time scale for the loss of spectral correlation, we use the spectral fluctuation correlation function,

$$C_{\chi_c \chi_c}(t) \equiv \langle \delta \chi_c(t) \delta \chi_c(0) \rangle$$

Any spectral inhomogeneity will result in a non-vanishing $C_{\chi_c \chi_c}(t \to 0)$. A typical spectral contrast fluctuation correlation function is shown in Figure 4.4. A non-vanishing correlation at $t \to 0$ is apparent and its decay can be described by a single exponential decay. This is in sharp contrast to time correlation traces from the almost perfectly spherical single silica beads and surface-immobilized gold nanoparticles both exhibit no detectable correlation. The time constant of the decay should depend on a nanoparticle’s size, as pointed out by the studies of Einstein [136]. However, the observable here is not the orientation of the particle, rather the spectral contrast determined by the orientation. To compare the
observed time constants to theories, we adopted Debye’s approach in dielectric relaxation. Under this Debye-Stokes-Einstein model, the relaxation time of \( l \)-th order spherical harmonics function is \( \tau_{l,DSE} = \pi \eta d^3/(l(l + 1))k_BT \), where \( \eta \) is the viscosity of the solvent, \( k_B \) is the Boltzmann constant, \( T \) is the temperature in Kelvin, and \( d \) is the hydrodynamic diameter of the particle. A rotation-coupled spectral fluctuation should exhibit a correlation time that is determined by the hydrodynamic diameter \( d \). To this end, we first used the nanoparticles 3D position trajectory as recorded by tracking (cf. inset in Figure 4.5a) to obtain the diffusion coefficient \( D \) [67, 107, 203]. We then used the Stokes-Einstein relation, \( D = k_BT/(3\pi \eta d) \), to calculate the hydrodynamic diameter. The fluctuation relaxation times of four batches of gold nanoparticles in water with nominal diameters of 80, 100, 150, and 200 nm, respectively, are plotted against the nanoparticles measured hydrodynamic diameters on a log-log plot in Figure 4.5a. Most of the data fall on the \( l = 2 \) line as predicted by the Debye-Stokes-Einstein DSE model for nearly spherical nanoparticles, \( C_{\chi_c\chi_c}(t) \propto \exp[-t/\tau_{DSE}^2] \) [124]. In general, the rotational relaxation for non-spherical nanoparticles should be an infinite sum of exponential decays over all \( l \) orders; however, higher-order terms tend to decay very rapidly in time due to the exponential dependence of the correlation function in \( l(l + 1) \) [124]. To a very good approximation, we found that a single exponential decay with \( l = 2 \) is sufficient for the fits with the current time resolution. These results thus further confirm that the spectral correlation can be used to quantitatively characterize rotational Brownian motion of single nanoparticles; therefore, the \( \chi_c(t) \) time trace cf.4.3d is a real-time realization of nanoparticles reorientation relaxation [204]. As a further verification, there should be analogous rotation-coupled fluctuations in the polarization of scattered light from nearly spherical nanoparticles. Polarization anisotropy has only been observed for single nanorods previously [205, 206] but has not been considered as a general feature of gold nanoparticles. Indeed, we have observed polarization anisotropy in individual nanoparticles. Figure 4.1g illustrates the experimental configuration for this experiment. The scattered light is decomposed into the vertical (V) and horizontal (H) polarization components. The polarization contrast is defined as \( \chi_p = (I_V - I_H)/(I_V + I_H) \). The autocorrelation function of the polarization fluctuation, \( \chi_p = \langle \delta \chi_p(t)\delta \chi_p(0) \rangle \), should also provide a measure for rotational dynamics that is consistent with and complementary to spectral fluctuation measurements. As shown in Figure 4.5b, the results can also be described well by the DSE model with \( l = 2 \) [124]. In both Figures 4.5 a and b, the non-spherical shape of nanoparticles may contribute to the deviation of the data points from the idealized DSE lines. Better experimental time resolution and more sophisticated theoretical treatments are expected to provide further insight for the shape dependent surface plasmon coupling as well as the nanoscale hydrodynamic interactions.

The spectral anisotropy of the surface plasmon resonance in metallic nanoparticles has long been neglected due to lack of experimental means to assess it. Single-particle tracking spectroscopy enables us to address this fundamental problem from a dynamical perspective, and the approach has been demonstrated here as a general tool to study dynamics of nanoparticles. In addition, the capability to correlate spectroscopy with the location of a moving nanoprobe could allow remote optical sensing of local temperature, pH, viscoelastic-
ity, and other parameters for the study of biological processes in cellular milieu, providing the much-needed contextual insights for a great number of problems in molecular biology. With the fast-advancing experimental innovations, we further anticipate, spectroscopy on single moving molecules will soon become practical, opening up new frontiers in the investigation of chemical reactivity in general.
Chapter 5

Observation of Correlated Emission Intensity and Polarization Fluctuations in Single CdSe/ZnS Quantum Dots

5.1 Introduction

Colloidal semiconductor nanoparticles, or quantum dots, have generated great interest because of their potential use as optical probes for sensing applications and also because of their unusual photophysical behavior. At the single-particle level, the emission intensity of quantum dots switches between periods of high ("on" state) and low ("off" state) intensity, termed blinking\cite{46}. The on/off time statistics of the blinking have been phenomenologically modeled by power-law distribution\cite{207,208}. This blinking of quantum dots has been shown to be correlated with fluctuations in the emission spectrum\cite{41,209,210} and lifetime\cite{211,212}. More recently, it has been shown that, rather than discrete on/off two states, there is a continuous distribution in the emission intensities and that the intensity is correlated with lifetime in a nonlinear way\cite{152}. In fact, this blinking phenomenon appears to be a general feature of emissive probes at the single-particle or single-molecule level and its underlying physical basis is an active area of research\cite{49}.

The current working hypothesis is that the luminescence quenching ("blink off") is due to charging in the quantum dot\cite{46,47}. Models ranging from trapped surface charges to various charge migration modes\cite{48,213–215} have been proposed to explain the dynamics of this time-dependent emission behavior of the quantum dots. In certain models, for example, the nanoparticle is considered to be in the (on) state when the charge is allowed to migrate on the surface, but the particle may ("blink off") when the charge(s) travels by a random walk process into the core. Other models based on the idea of diffusion-coupled electron-transfer mechanism predict differences in the photophysical behavior between the microsecond and
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millisecond time regimes. In addition to the excitation energy, the nanoparticle’s local dielectric environment has been shown to affect blinking of quantum dots. For example, the luminescence wavelength can be modified by the Stark effect. Taken together, these observations are consistent with a charged particle model. Along the same vein, localized charges are expected to mitigate a nanoparticle’s local dielectric environment such that the polarization of the emitted light, spectrum, and intensity should be correlated in some way. Although previous studies have reported that the time-averaged emission polarization for an immobilized particle does not change, it remains unknown if polarization fluctuates dynamically and correlates with luminescence intensity intermittency. It therefore will be of great interest to understand how, if at all, the intensity, luminescence lifetime, polarization, and spectral fluctuations are correlated in a time-dependent manner. The results will aid in gaining a more complete understanding of the fluctuations of optical observables that a single quantum dot can afford. In the present study, individual streptavidin-coated CdSe/ZnS quantum dots were examined with a confocal microscope with the capability to simultaneously measure the lifetime, intensity, color dichroism, and linear dichroism of the emission.

5.2 Methods

An amount of 20 µL of 0.1 nM solution of streptavidin-coated CdSe/ZnS core/shell quantum dots (Invitrogen lot no. 45024A) in isopropyl alcohol was spin-coated onto a quartz coverslip. A 0.1% (weight) poly(methyl methacrylate) (PMMA)/toluene solution was then spin-coated on top of the same coverslip. The coverslip was then placed on a modified Olympus IX70 confocal microscope similar to previous works. The sample was illuminated using 405 nm light from a frequency-doubled Ti:Sapphire laser (Tsunami, Spectra-Physics). A Pockel’s cell (M305, Conoptics) was set to pick every 32nd pulse reducing the repetition rate from 80 to 2.5 MHz prior to passing through a type-I BBO doubling crystal (Casix). The light was circularly polarized using a quarter waveplate (Tower Optical). Two band-pass filters (417/60, Semrock), a 25 µm pinhole spatial filter, and a series of neutral density filters were placed in the light path to block the 810 nm fundamental light and attenuate the beam. The average laser power prior to entering the microscope objective was 40 nW. Assuming a diffraction-limited focal spot at 405 nm and an 50% power reduction by the objective, the density is estimated to be 26 W/cm². In the current study, the excitation power density was purposely kept low in order to avoid multiphoton excitation processes and minimize known factors affecting the on-time statistics with high excitation power. The collimated light was then reflected off of a long-pass filter (488 DCXR, Chroma) before passing through a 1.3 NA infinity-corrected oil immersion objective (Olympus). The emitted light was collected by the same objective before passing through a 570 nm long-pass emission filter (E570LP, Chroma). A polarizing beam cube splitter (Newport) with an extinction ratio of 500:1 was used to separate the light into two orthogonal polarizations. The polarization beam splitter seen in Figure 5.1A serves two purposes: (i) it separates...
the light into two separate polarizations, and (ii) it serves as a linear polarizer to account for the polarization dependence of the dichromatic mirror. Note that the complications in quantifying polarization measurements through a microscope objective[124, 220, 221] do not play a significant role here because the present work focuses on the correlation between emission intensity and linear dichroism. The transmitted light through the polarization beam splitter was spectrally resolved by a dichromatic mirror (650DCRX, Chroma) with a band-edge empirically set at 658 nm (Figure 5.1B). The transmission spectrum of the dichroic was measured on the transmitted axis with a calibrated spectrometer. The light on each of the three axes were focused by a 75 mm lens onto three single photon counting avalanche photodiode modules (SPCM-AQRH-14, Perkin-Elmer) with measured response times of 680 ps at 405 nm (Figure 5.1A). The information from each of the detectors was sent through a discriminator, level translator, and delay lines (electronic components in Figure 5.1A, part l) before finally being collected by a time-correlated single photon counting card (SPM-600, Becker & Hickel), which recorded the absolute photon arrival time, the excitationemission delay time (microtime), and detector channel number. A piezoelectric stage with a range of 40 µm by 40 µm was used to raster scan the sample over the illumination volume. Raster scanned images of the quantum dots prior to data collection yielded a diffraction-limited spot on all three channels (Figure 5.1C). Once possible dots were identified, the diffraction-limited spots were centered over the illumination volume for time-dependent data collection for at least 5 min. A typical intensity-time trajectory is shown in Figure 5.2B. From the selected trajectories, the photon arrival times of all three channels were then analyzed using a change-point analysis algorithm[149, 152, 222] to detect and locate discrete intensity change points. Change-point segments, defined as the time trace between two successive intensity change points, were isolated for further analysis. The lifetime, color dichroism, polarization dichroism, and intensity were calculated over each segment. For each quantum dot, joint probability distribution maps weighted by the segment duration were calculated to examine the correlation among these four parameters.

5.3 Discussion

The fluctuations of the luminescence intensity, lifetime, spectrum, and polarization were studied using a time-resolved confocal microscope as has been discussed in a previous work.[152] The simultaneous examination of the polarization and spectrum observables was accomplished using three avalanche photodiodes (Figure 5.1A). In this configuration, the total intensity was calculated by considering the photons from all three detectors:

\[ I_{\text{total}} = I_1 + I_2 + I_3 \]  (5.1)

The linear dichroism and color dichroism were used to examine the polarization and spectral fluctuations, respectively. These were defined as

\[ \chi_p = \frac{I_1 - (I_2 + I_3)}{I_{\text{total}}} \]  (5.2)
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Figure 5.1: (A) Diagram of the experimental setup. The detector channel numbers are illustrated in the diagram. (B) Normalized transmission spectrum of dichroic in the microscope. The band-edge is located at 658 nm. The spectrum of the dichroic was normalized against the spectrum of the tungsten lamp measured in the absence of the dichroic. The maximum value of the transmission was then normalized to 1. (C) False color image of the diffraction-limited spot of the scanned particle. Scale bar is 300 nm. (D) Lifetime plot showing luminescence decay of the quantum dot (QD) at room temperature (blue) overlaid with instrument response function measured at 405 nm (red), full width half-maximum 680 ps. Reprinted with permission from Ref. [68]. Copyright 2008, American Chemical Society.

\[ \chi_c = \frac{I_2 - I_3}{I_2 + I_3} \]  

Therefore, \( \chi_c < 0 \) for red-shifted emission and \( \chi_c > 0 \) for blue-shifted emission. Once the microscope was centered over a particle of interest, the absolute photon arrival times, microtimes, and channel numbers were recorded. The durations between successively detected photons from all detectors were then processed using a likelihood ratio-based change-point segmentation algorithm.[149] The output of this algorithm is a series of photon indices corresponding to the most likely locations of where a change in the Poisson distribution of the photon arrival times has occurred. In the segments between two consecutive change points, the values of the linear and color dichroism were calculated. This approach has been used before to examine the correlation between the intensity and lifetime.[152] In the current configuration, the linear dichroism and color dichroism were correlated with the lifetime and total intensity.

In order to examine possible correlations between the different parameters, joint probability maps are constructed for each pair of parameters. The distribution plot represents a two-dimensional probability map between the lifetime and intensity calculated from the single-particle emission trajectories. The probability density correlating two parameters
Figure 5.2: (A) Typical quantum dot (QD) trajectory qualitatively displaying two dominant states. (BD) Respectively, values of the intensity, luminescence lifetime, color dichroism, and linear dichroism are calculated over time segments identified by change-point analysis and plotted as a function of time. Reprinted with permission from Ref. [68]. Copyright 2008, American Chemical Society.
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$p_1$ and $p_2$ is calculated by

$$P(p_1, p_2) = \frac{1}{t_{\text{total}}} \sum_{i=1}^{N} t_j \frac{1}{\sigma_{p_1,j}} \frac{1}{\sqrt{2\pi}} \exp\left[\frac{-(p_1 - p_{1,j})^2}{2\sigma_{p_1,j}^2}\right] \exp\left[\frac{-(p_2 - p_{2,j})^2}{2\sigma_{p_2,j}^2}\right]$$ (5.4)

where $N$ is the number of change-point segments, $t_j$ is the time duration of each change-point segment, $t_{\text{total}} = \sum_{j=1}^{N} t_j$, and $p_i$ and $\sigma_{i,j}$ represent the values of the parameters and their standard deviations, respectively, over the $j$th segment determined from change-point analysis. For the intensity and lifetime, the $\sigma_{i,j}$ are derived from the Fisher information expressions.[152] For the linear and color dichroism, the $\sigma_{i,j}$ are derived from propagating the error of the Poisson-distributed emission intensity uncertainties from the three channels. In constructing the probability distribution maps, the parameters over each segment are treated as Gaussian random variables. If the variables are uncorrelated then the resulting density map will either exhibit a horizontal line or a vertical line; deviations from these two limiting cases will be indicative of a correlation between the two parameters. A typical 5 ms binned intensity trajectory from the nanoparticles studied is shown Figure 5.2A. By eye, this trajectory shows the intensity switching between bright and dark states. Figure 5.2B-D show the form of the data, the parameters calculated as a function of change-point segment plotted versus time. The probability maps relating the four observables, lifetime, intensity, color dichroism, and linear dichroism are plotted in Figure 5.3A-D. Although there are considerable variations from quantum dots to quantum dots, there are some general features that are consistent throughout those studied. From the binned intensity trajectory in Figure 5.2A, one sees what appear to be two states. One would therefore expect to see two dominant populations in the correlations maps. In Figure 5.3, all the plots show evidence of two dense populations. A closer examination reveals that there is a continuous distribution of states connecting the two dominant populations, consistent with previous results.[152] Because the intensity has been shown to be correlated to the lifetime, the plots of lifetime versus color and linear dichroism have been omitted for clarity even though they show the same general continuous distribution.

In the present study, information about the lifetime, intensity, color, and linear dichroism of the emitted luminescence were recovered as a function of time on single dots. Consistent with the idea of a continuous distribution of intensities and lifetimes (cf., Figure 5.3A), parts B and C of Figure 5.3 show a continuous distribution of spectral and polarization shifts within the intensity trajectory from a single dot. That the high-intensity state appears to show a well-defined emission polarization can be attributed to the rodlike morphology of the quantum dot sample used,(21) which has been previously shown to have an aspect ratio close to 2.[152] Figure 5.3D further shows that the blue emission spectral shift is correlated with polarized emission. Note that when the particle is in its nonemissive state, only background and detector dark counts were recorded and therefore the signal, as expected, appears unpolarized. These observations are consistent with the idea of a charged dot can be emissive, but the physical location of the charge (most likely a hole state) can lead to a variety of emission characteristics.[210, 223] Within this framework, one expects that the migration of
Figure 5.3: (A) Joint probability map for luminescence lifetime vs intensity plot for the trajectory shown in Figure 5.2A. kcps stands for kilocounts/s. Consistent with previous results, there is a continuous distribution of intensity states. (B) Joint probability map for color dichroism and intensity. Red-shifting of the observed luminescence occurs continuously with decreasing intensity. (C) Joint probability map for linear dichroism and intensity. The degree of linear polarization of the emitted light occurs continuously with increasing intensity. The cartoons illustrate the current working model relating a charged quantum dot to its emission characteristics, in which the quantum dot becomes nonemissive only when the charge is localized at the CdSe core. (D) Joint probability map for color dichroism and linear dichroism shows two dense populations. In all plots, the darkest color represents the saturation value such that all probabilities greater than the threshold specified are given the same maximum value. Colors less than the saturation value are distributed linearly.

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a charge in the dot should mitigate the electric field to cause time-dependent fluctuations in polarization. In Figure 5.3, parts B and C, one sees continuous distributions of probability between intensity and the color and linear dichroism, respectively. In the figure, the probability plot shows increasing red-shifting of the spectrum with decreasing emission intensity. This is consistent with previous reports showing increasing red-shifting of the spectrum[224, 225] and concomitant red-shifting and darkening of the luminescence with an applied electric field.[225]

One of the complications in this experiment is the identification of single quantum dots using far-field optics; in particular, it has been shown using combined atomic force microscopy and optical detection that aggregates of quantum dots can display complex blinking statistics, whereas single quantum dots tend to show well-resolved two-state-like emission behavior.[226] The presence of single dots was inferred from the intensity traces which qualitatively show the presence of two dominant states (Figure 5.2A). The density maps in Figure 5.3A-D also show only two densely populated states with a continuous distribution of less populated states connecting the two (Figure 5.3A-D). From these data, the presence of a single dot within the diffraction volume is inferred. Future studies combining single-particle spectroscopy with simultaneous atomic force microscopy or some other imaging technique with higher spatial resolution should provide further insight on the nature of the emission and correlate them with the morphology of the particle. A careful study of the power dependence of the correlation between the different spectroscopic observables will also provide insightful information into the underlying mechanism.

In summary, the degree of emission polarization from individual quantum dots has been shown to correlate with the luminescence intensity. While the highly emissive states show highly polarized emission, the low-emissive states exhibit partially polarized light and correlate well with red-shifted spectrum and shortened luminescence lifetime—all are consistent with a model where variations of the local charge(s) around the quantum dot can lead to fluctuations in the local electric field. Understanding the fluctuations in the photophysical behavior of single quantum dots is important for such potential applications as probing and characterizing the chemical and physical properties of heterogeneous local environments, particularly how the environment may evolve over time.[88, 108, 227, 228] Future experiments integrating three-dimensional single-particle tracking[108, 116] with time-resolved studies maybe able to characterize the spectroscopic observables free of surface effects and provide a more detailed understanding of the correlation between the different spectroscopic observables presented in the paper.
Chapter 6

Luminescence lifetime characterization study of type Ia diamond nanoparticles

6.1 Abstract

Nanodiamonds are an interesting class of luminescent probe because of their resistance to bleaching or blinking. The source of nanodiamond luminescence is the color centers created by dopant impurities in various configurations. The H3 defect center, two nitrogens next to a vacancy, present in Type Ia diamonds has a potential to be used as a FRET donor because its green emission overlaps more favorably with the absorbance of larger range of organic dye molecules. This current study focuses on the study of the luminescent and spectral properties of individual 350 nm Type Ia nanodiamonds. The lifetime decay curves for individual nanodiamonds were fit to a stretched exponential function as opposed to single or double exponential fitting which has been done for studies of bulk Type Ia diamonds. The stretched exponential fitting indicates a distribution of lifetime components consistent with clusters of defect centers due to the aggregation of nitrogen impurities.

6.2 Introduction

In complex systems with internal dynamics spanning over multiple length and time scales, one seeks to understand how dynamics on the microscopic level lead to observed macroscopic phenomenology. In the cytoplasm of a cell for example, one might seek to understand how molecular crowding, causing changes in local viscosity and chemical behavior, affects biological activity. New experimental tools are required to probe a system over the required length and time scales in order to answer these types of questions. Spectroscopy is a powerful means to interrogate the dynamics in these kinds of systems, but available nanoscopic optical probes have non-trivial photo-physical limitations.
In Fluorescence Resonance Energy Transfer (FRET) one fluorescent or luminescent probe, termed the donor, is excited by a light source then energy is transferred to a secondary probe, termed the acceptor. Because the rate of energy transfer is related to the $1/r^6$ [66], FRET can be an accurate measure of distance changes between donor and acceptor. When the distance is fixed, spectral shifts in the emission spectra of the donor or absorption spectra of the acceptor can cause changes in the rate of energy transfer. This idea has led to the development of FRET-based chemical sensors [39, 150, 229–232]. Quantum dots (QDs) and organic dyes are some of the most commonly used probes for FRET imaging and chemical sensing [39, 66, 229–232]. However, their photo-stability problems are well documented. Quantum dots display power-law dependent [150, 207, 208] intensity fluctuations [46, 49, 208], requiring advanced statistical methods to be used for quantitative single-molecule FRET applications [89]. Organic dyes are also susceptible to bleaching (typically yielding $10^6$ photons) and well as blinking [46, 233]. These issues not only limit the amount of physical information that can be extracted, but also restrict the time and length scales of observation.

Nanodiamonds are a class of luminescent probe known not to bleach or blink. These composed of small crystals of carbons classified into several types according to features in their IR and UV absorption spectra [89, 234]. These features have been attributed to different dopant and vacancy configurations within the crystal, termed defect centers. Because nanodiamonds are known not to bleach or blink, the physics of defect centers in diamonds has become an interesting research topic. They have also drawn attention due to their low cytotoxicity [234, 235], and potential for quantum information processing applications [236, 237]. Although some research has been done on the optical properties of (N-V)- centers in type Ib [235, 237] and H3 centers in type Ia diamonds [238] on the nanometer scale, early work on the study of nanodiamond defects focused predominantly on the properties of bulk diamonds [239–246]; however, the spectroscopic properties of many materials are different on the bulk and nanometer scales. For example, the absorption and scattering properties of metallic nanoparticles change depending on particle size and shape [247, 248]. In quantum dots, as the particles become larger or more aspherical the emission wavelength can shift [249]. It has also been shown that the surface tension of quantum dots can alter its spectroscopic properties [250]. In order to be used for FRET, the properties of the H3 defect center in Type Ia diamonds needs to be examined on the nanometer scale.

Nearly all synthetic diamonds are classified as type Ib diamonds. Nitrogen impurities are evenly distributed as singly substituted atoms and form a negatively charged nitrogen-vacancy center, (N-V)-, [234] which also can be produced artificially by thermal annealing of irradiation damaged type Ib diamond [238]. The zero phonon line of Type Ib diamonds is 637 nm yielding red emission [239]. Although (N-V)- centers in Type Ib diamonds have been the most extensively studied defect center in diamond, the green luminescence from the H3 defect center present in Type Ia diamonds has the advantage of overlapping with the green absorption of many organic dyes. Nearly all natural diamonds are classified as type Ia diamonds because of a relatively large nitrogen content, which is present as clusters or aggregates [240, 241]. Other defects such as the (N-V)- center are present in irradiated Type Ia diamonds. The H3 defect, caused by the replacement of two adjacent carbons with
nitrogens next to a vacancy (Figure 1), has a zero-phonon line (ZPL) peaking at 503nm \[242\] yielding green luminescence emission. Because of their remarkable photostability and long luminescence lifetime, nanodiamonds could potentially serve as FRET donors. Type Ib nanodiamonds have even been proposed for single-molecule FRET applications \[243\]. Other luminescent probes such as quantum dots \[244, 246, 251\] and lanthanides \[247–249\] have been used as FRET donors. Their longer lifetime allows for a faster rate of energy transfer and greater likelihood the relative orientation of the donor and acceptor will achieve the fully randomized orientation parameter value of 2/3 \[66\].

Early studies of the luminescence lifetimes of H3 and (N-V)- defects found the H3 lifetime to be 16.5 ns \[250\], 12.9 ns for (N-V)- centers under flash lamp excitation \[239\], and 6.49 ns for (N-V)- centers under picosecond illumination \[252\]. The optical properties of (N-V)-centers in type Ib diamonds have been characterized in bulk and on the nanometer scale \[253, 254\]. These early works predominantly focused on the properties of bulk diamonds; however, the spectroscopic properties of many materials are different on the bulk and nanometer scales. For example, the absorption and scattering properties of metallic nanoparticles change depending on particle size and shape \[185, 186\]. In quantum dots, as the particles become larger or more aspherical the emission wavelength can shift \[255\]. It has also been shown that the surface tension of quantum dots can alter its spectroscopic properties \[256\]. In order to be used for FRET, the properties of the H3 defect center in Type Ia diamonds needs to be examined on the nanometer scale.

In the current study, the power dependence of the lifetime, intensity, spectral shift and polarization 350nm Type Ia nanodiamonds diamonds were characterized. It was found that the optical properties of the nanodiamonds were largely independent of excitation power and there was not any detectable correlation between the intensity, lifetime, spectral shift or polarization unlike other imaging probes \[68, 152, 252, 253\]. Unlike previous reports which used a single or double exponential model for the luminescence decay, a stretched exponential model was used. Large variation in the fitted stretching exponent, and decay lifetime indicate a distribution of compositions within the particle sample.

### 6.3 Experimental Procedures

The 350 nm Type Ia diamond samples were obtained from Microdiamond (Switzerland) and prepared using published protocols \[238, 257\]. The experimental procedure for simultaneously measuring the lifetime, spectral shift and polarization is similar to earlier publications \[68, 152, 252, 253\] but with a few modifications (Figure 2). Briefly, 45\(\mu l\) of a 200\(\mu g/ml\) solution of 350nm type Ia nanoparticles in deionized water was spin-coated on a quartz coverslip. Figure 2A shows the diagram of the experimental setup. The quartz coverslip was illuminated with circularly polarized 405nm light from a frequency-doubled Ti:Sapphire laser (Tsunami, Spectra-Physics) with a repetition rate of 2.5 MHz. The average laser power densities at the focus of the microscope objective were 3\(KW/cm^2\), 29.3\(KW/cm^2\), 97\(KW/cm^2\) assuming a 50% power reduction by the microscope objective. The emitted light
Figure 6.1: Normalized excitation and emission spectrum of 350nm type Ia diamond nanoparticles obtained with SpectraMax M5 (Molecular Devices) spectrometer. Above the spectrum, there are three schematic drawing represent different color centers, N3, H3 and (N-V)- centers. The filled circles (black) represent nitrogen; filled circles with grey color are carbon, and open circles represent vacancies. The dotted line (marked with ∆) to the left is the excitation spectrum of H3 center, and the solid line marked with ∆ is the emission spectrum of H3 center. From the H3 center emission spectrum, the peak wavelength is around 530nm, and the excitation scan was obtained by fixing the emission wavelength at 530nm, the obtained excitation peak wavelength is around 450nm. The arrow pointed at 405nm illustrates the wavelength position of the excitation wavelength of our laser system. 530nm with an additional broad band spanning from 600-800nm contributed by the (N-V)- centers. The dotted line (marked with open circle) in the middle is the excitation spectrum of (N-V)- center, and the solid line marked with open circle is the emission spectrum of (N-V)- center. From the (N-V)- center emission spectrum, the peak wavelength is around 650nm, and the excitation scan was obtained by fixing the emission wavelength at 650nm, the obtained excitation peak wavelength is around 550nm.
CHAPTER 6. LUMINESCENCE LIFETIME CHARACTERIZATION STUDY OF TYPE IA DIAMOND NANOPARTICLES

Figure 6.2: (A) Diagram of the experimental setup. The recollected light is initially split by a polarizing beam cube splitter and reflected onto detector 1 while the transmitted light is split by a dichromatic mirror to isolate the contributions from the H3 and (N-V)- defect centers. (B) the normalized transmission spectra of the dichroics and emission filters used in the experiment are overlaid on top of the bulk luminescence spectra for the H3 and (N-V)-defects.

was recollected by the same 0.7 NA objective and filtered using a 488nm Raman filter (LP03-488RS-25, Semrock) and a 500-700nm band pass filter(E480LP Chroma). The light on the reflected axis of a polarizing beam cube splitter (Newport) then passed through a long-pass dichromatic mirror (DC570LP, Chroma) with a band-edge empirically set at 565nm. An emission filter (LP570 Chroma) was also placed on the transmitted axis (Figure 1B). Using this configuration, the light from the H3 and (N-V)- defect centers were collected on the reflected light and transmitted light of the dichromatic mirror. The instrument response times on all three avalanche photodiodes (SPCM-AQRH-14, Perkin-Elmer) was less than 650ps at 405nm. Figure 3A compares typical decay profiles for the instrument response, nanodiamond luminescence and non-luminescent background. Chi-squared fitting was done by convolving the instrument response with a model stretched exponential model in order to fit it to the observed decay. Typical decay curves for the instrument response, non-luminescent background and luminescent decay for the 350 nm particle samples are plotted in Figure 3A.
6.4 Results and Discussion

FRET can be used to make sensitive, powerful spectroscopic rulers and chemical sensors. A major challenge is developing photo-stable probes to provide long time information for the study of complex systems. This is one motivation for the use of lanthanides and quantum dots as both FRET donors [230, 247, 248] and acceptors [258]. However, lanthanides and quantum dots are known to suffer from cytotoxicity issues. Nanodiamonds, however, have shown to be photo-stable even under high excitation power and also biocompatible [233–236].

As the mean lifetime increases, the system stays in an excited state longer allowing for increased energy transfer and increased time for the donor and acceptor orientations to randomize. Although most work on applications of nanodiamonds has focused mainly on the red luminescent (N-V)- defect centers of Type Ib diamonds. The green luminescent H3 defect centers in Type Ia nanodiamonds could potentially be useful as FRET donors for a wide variety of green absorbing acceptors. The luminescence decay time of H3 and (N-V)- centers has been measured and published in previous publications and fit using a discrete number of states. Early studies of the luminescence lifetimes of H3 and (N-V)- defects found the H3 lifetime to be 16.5 ns [251], 12.9 ns for (N-V)- centers under flash lamp excitation [240], and 6.49 ns for (N-V)- centers under picosecond illumination [246]. In this report, the luminescence decay of the Type Ia nanodiamonds was examined and found
to fit to a stretched exponential model with a large range of values for the fitted stretching exponent, $\beta$, and luminescence decay lifetime, $\tau$. Unlike (N-V)- centers which can be created using a high energy electron beam in a low enough density to observe single centers, the defect centers in the 40 KeV He$^+$ irradiated Type Ia nanodiamonds have domains of defect centers caused by the inhomogeneous aggregation of nitrogen. Empirically, a distribution of states is observed. A typical fit of the stretched exponential model to the luminescence decay is plotted in Figure 6.3B. This function, first used to empirically describe electrical discharge in a leiden jar [254, 259], has also been used to describe, structural relaxation [185, 260], dielectric relaxation [186, 255, 261, 262], and lifetime decays [256, 263] etc. As opposed to assuming a discrete number of lifetime components, the stretched exponential function can be used to describe a continuous distribution of states [68, 152, 264, 265].

$$\phi(t) = \exp[-(t/\tau_{kww})^{\beta_{kww}}] = \int_0^{\infty} \exp[-t/\tau] \rho_{kww}(\tau) d\tau \quad (6.1)$$

Here, $\rho$ represents a continuous distribution of lifetimes. The distribution function is related
Figure 6.5: The fitted lifetime parameter $\tau_{kww}$ is plotted for the red and blue channels at each of the power densities used in the experiment. The fitted lifetimes spanned several orders of magnitude consistent with the large variation of stretching exponents and spectral shifts observed in this experiment.

to the stretched exponential by an inverse Laplace transform [257, 266].

$$\rho_{kww}(\tau) = \left(\frac{1}{\tau^2}\right) L^{-1}[\phi(t)]$$

$$= -\frac{\tau_{kww}}{\pi \tau^2} \sum_{k=0}^{\infty} \frac{(-1)^k}{k!} \sin(\pi \beta_{kww} k) \Gamma(\beta_{kww} k + 1)(\frac{\tau_{kww}}{\tau})^{\beta_{kww} k + 1}$$  \hspace{1cm} (6.3)

A common convenient way to understand the physical significance of the distribution $\rho_{kww}(\tau)$ is to remove the explicit dependence on the lifetime, $\tau$, using

$$G(\frac{\tau}{\tau_{kww}}) = \tau \rho_{kww}(\tau)$$  \hspace{1cm} (6.4)

When one plots $G(\frac{\tau}{\tau_{kww}})$ versus $\ln(\frac{\tau}{\tau_{kww}})$, one gets a sense of the width of the distribution. As $\beta$ increases, the width of the distribution function becomes narrower. $\beta$, the stretching exponent, takes on values from 0 to 1. When the stretching exponent takes a value of 1, then the form for the intensity distribution becomes a single-exponential decay. The value of the stretching exponent therefore controls the shape of the distribution. The application of the stretched exponential distribution has appeared before in early FRET literature to describe the donor intensity in the presence of energy transfer [258, 267]. The generalized expression
for donor intensity distribution in this case is,

\[ I(t) = \exp\left[\frac{-t}{\tau} - A(\beta)\left(\frac{t}{\tau}\right)^\beta\right] \] (6.5)

where the value of \( \beta \) will take on values of 1/2, 3/8 and 3/10 for dipole-dipole, dipole-quadrupole, or quadrupole-quadrupole interactions [258, 267–269]. Here, the distribution of lifetime states was attributed to the presence and interaction of multiple defect centers, dominated by the H3 and (N-V)- centers in varying concentrations. For 40 KeV He+ irradiation, the concentration of defect centers is 10 ppm [238, 257], corresponding to several thousand defect centers within one 350nm nanodiamond. The experimentally fit stretching exponents, ranging from 0.2 to 1, indicate a great deal of heterogeneity in the composition of the individual nanodiamonds.

The main feature of the fitted lifetimes, plotted in Figure 6.4, is the way they are spread over several orders of magnitude, indicating large particle to particle variation. One can understand the fitted lifetime parameter \( \tau_{kww} \) in terms of the ratio \( \frac{\tau}{\tau_{kww}} \). The peak values of the lifetime distributions in figure 6.4A are centered near where the ratio \( \frac{\tau}{\tau_{kww}} = 1 \). The specific fitted value of shifts the lifetime distribution around but does not change the shape of the distribution it only rescales and shifts it. The large range in fitted parameters suggests an upper bound on the domain size for these heterogeneities. The size of these domains must necessarily be smaller than the particle size in order to observe a high degree of particle to particle heterogeneity. This physical picture of different, possibly interacting, states may also be complicated by differences in surface tension, arising from particle shape diversity, altering the properties of surface defects [270, 271]. It is important to note that for FRET applications the rate of donor recombination should be slower than the rate of energy transfer. The observed lifetimes span several orders of magnitude and it has been shown in systems with quantum dot FRET acceptors a too fast donor lifetime prevented the observation of FRET [258, 272].

More than just interesting luminescence decays, diamonds have a high index of refraction \( n = 2.42 \), suggesting fluctuations in scattered light can be used to measure the local viscoelastic response. The spectral and polarization fluctuations from metallic nanoparticles can be used to measure their rotational diffusion [106, 259]. The experimental design, illustrated in Figure 6.3A, uses three detectors to simultaneously measure spectral and polarization fluctuations similar to previous reports. The light collected from the fluorescence of a single nanodiamond is recollected and then split by a polarizing and then again by a dichromatic mirror. The transition edge of the dichromatic mirror was chosen to split the spectral contributions from the H3 and (N-V)- centers (figure 6.2B). The linear and color
dichroisms, defined by

\[ \chi_p = \frac{I_1 - (I_R + I_B)}{I_1 + (I_R + I_B)} \]

\[ \chi_c = \frac{I_R - I_B}{I_R + I_B} \]

are a way of measuring the degree of polarization and spectral shift. The values of \( \chi \), plotted in Figure 6A, show no net polarization because the values are distributed around 0. There is also no induced polarization at higher powers. Figure 5B shows the values of centered on some non-zero value which is to be expected because the spectrum for the nanodiamonds may not be perfectly centered over the transition edge of the dichromatic mirror for every nanodiamond. The variation in values of \( \chi \), is consistent with the heterogeneous composition of defect centers leading to a variety of spectral shifts. No statistically significant cross-correlation \([260, 273]\) was observed between the polarization and color fluctuations suggesting the source of the emission is not fluctuating dynamically within nanodiamond. This observation is consistent with the idea that laser-induced migration \([261, 262, 274, 275]\) of the nitrogen vacancies is not significant.

In this report, the luminescence emission of the nanodiamonds yielded a distribution of lifetime states, presumably due to irradiation with the He+ beam needed to create the defects. Given the large number of defect centers, one might think spatial averaging the spectral components would produce homogeneous properties. Instead, the large variation in stretched exponential fitting parameters, luminescence intensities, and color dichroism values indicate the presence of clusters of defect centers on the nanometer scale. FRET using a donor with stretched exponential intensity distribution has been discussed by Felorzabihi et al. in the context of a donor organic dye molecule in a polymer film \([263, 276]\). In their experiments, a donor dye molecule was placed in a polymer film causing a stretched exponential intensity profile in the absence of any acceptor molecules. One of the main difficulties found in applying Type Ia nanodiamonds for FRET is the necessity for accurate determination of the mean radiative lifetime and Frster radius, \( R_0 \) which is dependent on the degree of spectral overlap between donor and acceptor. Since each nanodiamond is heterogeneous in composition leading to a high degree of variation in lifetimes and spectral shifts, the same nanodiamond used for characterization must also be the one used in the experiment. This experimental hurdle could possibly be overcome by using lower dose irradiation or using smaller particle samples to create more homogeneous particle samples. Further research would not only benefit FRET applications but others such as quantum information processing applications requiring single photon sources.
Chapter 7

Ligand Synthesis and Passivation for Silver and Large Gold Nanoparticles

7.1 Introduction

Materials on the nanometer scale can exhibit optical properties different than the corresponding bulk materials. For noble metal nanoparticles, the source of the size-dependent optical response is the collective oscillation of conduction-band electrons in response to an applied electromagnetic field, \( i.e. \), the particle’s plasmon resonance [53, 277, 278]. In the near-field region (distance to particle surface \( \ll \) wavelength), nanoparticles can enhance the local field leading to emission enhancement for emitters present in the field. The plasmon resonance and near-field distributions can be tuned using differently shaped nanoparticles or hierarchical structures composed of multiple particles.

Noble metal nanoparticles, with plasmon resonances in the visible spectrum, have attracted a lot of attention in the literature for a variety of applications. The synthesis of these particles typically involves the reduction of the metal ions out of solution leading to Ostwald ripening of seed particles [279]. Particles are typically ripened to a desired size and then stabilized with a surfactant.

The advantageous optical properties and the known surface chemistry of these nanoparticles have led experimenters to conjugate them to biological materials for a variety of applications. One can attach small molecules to the nanoparticle surface to prevent non-specific adsorption of biomolecules or attach molecules with an orthogonal chemistry for hierarchical construction. Applications of these hybrid particles range from analyte detection [280], electron microscopy contrast agents [281], photo-thermal therapies [282, 283], drug delivery systems [284], bio-barcoding [285, 286], among others [287–291].

For certain applications, \( e.g. \) single-particle-based sensing and spectroscopy, using particles with increased scattering cross-sections such as larger-diameter gold nanoparticles may be desirable. Utility of any hybrid nano-material relies on the ability to produce stable colloids resistant to aggregation and non-specific adsorption of biomolecules under physio-
logically relevant conditions. As the particle size becomes greater, however, the increased susceptibility to surface oxidation and the larger surface area per particle present challenges to robust incorporation of these particles into the optical toolbox of nano-materials [292–295].

This chapter details the practical procedures that have been routinely used in our laboratory for the preparation of nanoparticles for biological applications. Following the list of materials and equipment below, the remainder of the chapter is divided into three parts: The first section describes the synthesis of small molecules for the passivation and bio-conjugation of silver and large gold nanoparticles. The second section describes the passivation of silver and large gold nanoparticles. The last section describes methods to characterize the effectiveness of the passivation.

### 7.2 Materials and Equipment

1. 18.2 MΩ-cm water
2. room temperature is 20 °C
3. \((\pm)\alpha\text{-lipoic acid (LA), }\geq 99\% \text{ (Sigma-Aldrich)}\)
4. thionyl chloride (SOCl₂), 99 + % (Alfa Aesar)
5. 3-dimethylamino-1-propanol, 99% (Alfa Aesar)
6. 1,3-propanesultone, 99% (Alfa Aesar)
7. sodium borohydride (NaBH₄) (Fisher)
8. chloroform (CHCl₃), ACS grade (EMD)
9. acetone, HPLC grade (Sigma-Aldrich)
10. sodium bicarbonate (NaHCO₃)
11. ethyl acetate (EtOAc), ACS grade (EMD)
12. pasteur pipette
13. cotton balls
14. agarose, bioreagent grade(Fisher)
15. New England Biolabs Tridye 1kb DNA ladder
16. ethanol, 200 proof (Pharmco-AAPER)
17. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), stored in a desiccator at −20 °C, (Thermo Scientific)

18. sulfo-NHS, stored in a desiccator at 4 °C (Thermo Scientific)

19. ethylenediaminetetraacetic acid (EDTA), bioreagent grade (Fisher)

20. 0.1 M MES (2-(N-Morpholino)ethanesulfonic acid sodium salt) pH 6, bioreagent grade (Fisher)

21. Sephacryl S-300, size exclusion resin (GE Lifesciences)

22. chromatography media sampler pack (Bio-Rad)

23. mini-tube rotator

24. rotary evaporator

25. 80 nm gold nanoparticles (British BioCell International)

26. Speed-Vac, i.e. Savant SVC-100 or equivalent

27. aluminum foil

28. Schlenk line

29. sodium acetate, bioreagent grade (Fisher)

30. silver nitrate, 99.9999% (Sigma-Aldrich)

31. sodium citrate dihydrate, granular/certified (Fisher)

32. L-ascorbic acid, ACS grade (Fisher)

33. sodium hydroxide, ACS grade (Fisher)

34. dithiolalkanearomaticPEG6-NHS (CAS# 936115-55-8, SensoPath Technologies); the solution is extremely susceptible to hydrolysis, store at −20 °C and use immediately after opening. Do not store unreacted stock solutions.

35. EZ-Link Amine-PEG2-Biotin (Pierce)

36. 10 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) pH 8, bioreagent grade (Fisher)

37. temperature-controlled water bath

38. three-neck jacketed round bottom flask
7.3 Small-Molecule Ligand Preparation for Large Gold Nanoparticles

The passivation of bare metal nanoparticle surfaces with organic ligands is important for colloid stability and minimizing non-specific adsorption of biomolecules. The attachment chemistry to the metal nanoparticle [296], coverage efficiency [297, 298], ligand length [299], and terminal group are all important control parameters [300].

Thiol groups are commonly used for attachment of small molecules to noble metal nanoparticles because chemical bonds are formed with the metal surface [301]. To reduce ligand lability and reduce susceptibility to oxidative cleavage, dithiol groups have been used to increase coordination to the gold surface [296, 302, 303].

The ligand’s terminal group can serve multiple roles. One of these is to increase steric and electrostatic repulsion between particles to increase colloid stability. Zwitterionic ligands, although uncharged, form a charged double layer around the nanoparticle and have been shown to yield stable colloids [304–308] under physiologically relevant conditions.

Another role for the terminal moiety is to provide an orthogonal chemistry for rational construction of biological or inorganic nanoparticles. Streptavidin, a 53 kDa tetrameric protein, binds strongly to biotin [309] and has been used to construct bio-inorganic composites for various applications [310–312].

The following section describes the synthesis of three ligands for particle passivation. The first is a compact zwitterionic ligand containing a sulfobetaine moiety derived from lipoic acid. The second is a dithiol ligand with a biotin terminal group, enabling the bio-conjugation to streptavidin modified species. The third procedure describes the modification of single stranded DNA with lipoic acid, for direct bio-conjugation of DNA onto larger gold nanoparticles.

Protocol for Dihydrolipoic Acid - Sulfobetaine (DHLA-SBE) Synthesis

1. All reactions performed under N₂ with a Schlenk line, unless otherwise stated.
2. Dilute lipoic acid (1000 mg, 4.85 mmol) in 10 mL CHCl₃ and cool in an ice bath.
3. Dissolve SOCl₂ (5.34 mmol) in 2-3 mL CHCl₃ and add drop-wise.
4. After addition of SOCl₂, stir 30 minutes at room temperature.
5. Remove the solvent by rotary evaporation.

6. Re-dissolve the product in 10 mL CHCl₃, cool in an ice bath and purge with N₂.

7. Dilute 4.85 mmol 3-dimethylamino-1-propanol with 2-3 mL CHCl₃ and add drop-wise.

8. Stir the reaction vigorously overnight at room temperature.

9. Quench the reaction with NaHCO₃ (9.7 mmol) in 20 mL water.

10. Extract the quenched reaction with EtOAc (2x) and wash the combined organic extract with saturated aqueous NaHCO₃ (2x).

11. Combine organic layers, dry over MgSO₄ and filter.

12. Remove the solvent by rotary evaporation.

13. Dissolve the product in 10 mL acetone.

14. Dissolve 4.85 mmol 1,3-propanesultone in 2-3 mL acetone and add drop-wise at room temperature.

15. Reflux the reaction at 75 °C overnight under N₂. The final product will precipitate out of solution.

16. Filter the precipitate and wash with CHCl₃. Dissolve the product in water and extract the aqueous layer with CHCl₃. The desired product will be in the aqueous layer.

17. Filter through a pasteur pipette with a cotton plug and evaporate under reduced pressure.

18. Characterize the product. ¹H NMR (500 MHz, DMSO-d6): δ [ppm]: 3.46-3.50 (t, 2H), 3.38-3.43 (m, 2H), 3.28-3.35 (m, 2H), 2.98-3.06 (s, 6H), 2.77-2.85 (m, 2H), 3.46-3.50 (m, 4H), 2.19-2.23 (t, 1H), 1.87-2.08 (m, 4H), 1.79-1.86 (m, 2H), 1.27-1.63 (m, 6H). ESI MS m/z: 414.14451 (M+H)+.

19. Reduce dithiol bond with an equimolar amount of NaBH₄ just before use.
20. Store unreduced product as a solid in $-20^\circ\text{C}$ freezer until needed. The structure shown in Figure 7.3 is of the product after reduction of the disulphide bond with NaBH$_4$.

**Protocol for Dithiol-biotin Synthesis**

1. Store 10 mg (12.6 $\mu$mol) dithiolalkanearomatic PEG6-NHS ligand at $-20^\circ\text{C}$, unopened in a dessicator until required.

2. Dissolve 6 mg (16 $\mu$mol) EZ-Link Amine-PEG2-Biotin in 500 $\mu$L 10 mM Hepes buffer at pH 8.

3. Add solution *immediately* to freshly opened bottle of dithiolalkanearomatic PEG6-NHS. The NHS ligand is very reactive and will hydrolyze quickly if not used immediately.

4. Let mixture react for 2 hours at room temperature.

5. Store the final solution (dithiol-biotin) at $-20^\circ\text{C}$.

**Preparation of Lipoic Acid Functionalized Single Stranded DNA**

The following protocol uses EDC chemistry for functionalizing aminated single-stranded DNA (ssDNA) with lipoic acid. The relatively low cost of aminated primers allows for the increased preparative scale necessary to passivate large gold nanoparticles with DNA. Here, amine 5'-modified ssDNA is coupled to LA and then a complementary strand, 5'-modified with biotin, is annealed to the LA-modified strand.

1. Prepare 10 mg/mL solution of lipoic acid in DMSO.

2. Equilibrate EDC and sulfo-NHS to room temperature.

3. Add 360 $\mu$L of 10 mg/mL lipoic acid (0.0174 mmol) solution to 640 $\mu$L 0.1 M MES at pH 6.

4. Add aqueous lipoic acid solution to an Eppendorf tube containing 14 mg (0.073 mmol) EDC and 22 mg (0.1 mmol) sulfo-NHS.

5. Let mixture react 5 minutes at room temperature to activate carboxylic acid.

6. Prepare 500 $\mu$L 0.5 mM aminated DNA in water and add to activated lipoic acid mixture.

7. Rotate on a mini-tube rotator at room temperature for 2 hours.

8. Ethanol precipitate DNA using 1 mL 0.3 M sodium acetate in ethanol.
9. Incubate at 4 °C for 1 hour.

10. Spin at 13,000 rpm for 1 hour at 4 °C in a microcentrifuge.

11. Carefully discard the supernatant.

12. Add 500 μL 70% ethanol, cooled to 4 °C.

13. Centrifuge the solution at 13,000 rpm for 20 minutes at 4 °C.

14. Carefully discard the supernatant.

15. Dry the DNA pellet with a Speed-Vac.

16. Dissolve the pellet in 50 μL 10 mM Hepes buffer at pH 8.

17. Quantitate the DNA by absorbance at 260 nm.

18. Dilute complementary ssDNA, 5'-modified with a biotin, in 10 mM Hepes buffer at pH 8.

19. Add an equimolar amount of complementary ssDNA to lipoic acid-functionalized single-stranded DNA.

20. Add sodium chloride to a concentration of 100 mM.

21. Heat DNA to 95 °C for 2 minutes.

22. Cool DNA on bench top at room temperature.

23. Store the lipoic acid-DNA at −20 °C until needed.

### 7.4 Particle Passivation with Dithiol Ligands

Methods for the attachment of biological materials to noble metal nanoparticles are mature enough to allow the synthesis and purification of discrete 1-1 bio-conjugated nanoparticles [313]. The more general topic of interfacing biological molecules with nanoparticle surfaces has been reviewed previously [314, 315]. The majority of these bio-conjugation methods, however, have been applied to smaller-diameter gold nanoparticles (diameter < 40 nm). The increased surface area per particle for larger diameter noble metal nanoparticles presents challenges for non-specific absorption and colloid stability [294, 295]. In this section, a procedure for passivating large gold nanoparticles is presented.
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Large Gold Nanoparticle Passivation

1. Thaw DHLA-SBE from section Section 7.3, dithiol-biotin from Section 7.3, and lipoic acid-DNA from Section 7.3 to room temperature.

2. Add 10 µL of 50 mM DHLA-SBE to either 1 µL of 10 mM dithiol-biotin or 10 µL of 500 µM lipoic acid-DNA.

3. Add an equimolar amount of NaBH₄ to the mixture and incubate 5 minutes.

4. Add reduced thiols to 500 µL of 80-nm gold nanoparticle(10¹⁰ particles/mL).

5. Rotate the mixture on mini-tube rotator overnight at room temperature.

6. Save an aliquot of mixture for characterization by gel electrophoresis and uv-vis absorption spectroscopy.

7. Dialyze gold nanoparticles in a 300-kDa membrane for 2 hours at room temperature in 4 L water.

8. Exchange the 4 L water every two hours for a total of 3 exchanges.

Silver Nanoparticle Synthesis and Passivation

Colloidal silver nanoparticles have an exceptionally strong and sharp plasmon resonance useful for different applications. Due to their utility, there have been numerous synthetic procedures using different reducing agents, such as citric acid [316], ascorbic acid [317], sodium borohydride [318], and hydrogen gas [319]. On the other hand, silver nanoparticles are even more susceptible to surface oxidation than gold nanoparticles, thereby impacting on the proper preparation for biological applications. This can be overcome by synthesizing the desired silver nanoparticles in house (rather than purchasing them from commercial sources) and passivating the freshly synthesized particles immediately. Once the silver nanoparticles are properly passivated, they can be stably stored for longer period of time and still retain the reactivity for further bioconjugation. In the following section, silver nanoparticles are synthesized using a modified ascorbic acid reduction method and then passivated with dithiol ligands.

1. Prepare 20 mg/mL silver nitrate stock solution fresh for each synthesis.

2. Prepare 70 mg/mL trisodium citrate, 2 mg/mL ascorbic acid, and 0.1 N NaOH stock solutions.

3. Connect a 500 mL three-neck jacketed round bottom flask to a circulating water bath set to 75 °C.
4. Wrap flask with aluminum foil. An example synthesis apparatus is depicted in Figure 7.2.

5. Add 100 mL of ultra-pure 18.2 MΩ-cm water along with a stir bar.

6. Center the flask on a stir plate set to 750 rpm.

7. Cap the open necks of the flask with rubber stoppers.

8. Load two syringe pumps with NaOH and ascorbic acid stock solutions. Set syringe pumps to 250 µL/min for two minutes of NaOH addition, and 2 mL/min for five minutes of ascorbic acid addition.

9. Once the water reaches 75 °C, equilibrate for five minutes before adding reactants.

10. Quickly add 1 mL of silver nitrate. Wait 5-10 seconds, then quickly add 1 mL of trisodium citrate stock solution by syringe.

11. Add 500 µL of NaOH at 250 µL/min for 2 minutes and 10 mL of ascorbic acid at a rate of 2 mL/min for a total of five minutes.

12. Remove any injection needles and let react at the set temperature for 30 minutes.

13. Store product at 4 °C.

14. Spin 10 mL of 0.5 nM silver nanoparticles at 12,125 rpm for 30 minutes.
15. Remove the supernatant and re-suspend the pallet in methanol.
16. Add DHLA-SBE to a final concentration of 500 mM.
17. Rotate on a mini-tube rotator for 24 hours at room temperature.

7.5 Evaluation of Passivation Effectiveness

For self-assembled monolayers on flat surfaces there exist techniques such as ellipsometry [320] and quartz crystal microbalance [321] measurements to quantify surface coverage. Nanoparticles typically display particle-to-particle variation; the extent of surface coverage [298, 322] and non-specific adsorption [315] need to be characterized to understand the scope of a new particle system. Ideally one would desire the ligands to be resistant to non-specific adsorption and dissociation from the particle surface while also being bio-conjugatable. In the following section, particles are characterized by absorption spectroscopy, gel electrophoresis, and column chromatography to ensure the robustness of the passivation. It should be pointed out that the latter two characterization methods are routinely used in preparing proteins and nucleic acids; ensuring that the passivated gold and silver nanoparticles are compatible with these standard biochemical methods is a necessary step to enable subsequent manipulation of the desired hybrid nano-materials.

Absorption Spectra of Large Gold Nanoparticles

1. Record uv-vis absorption spectrum for each particle sample before and after dialysis. If the particles are stable and monodisperse, they should still retain their characteristic plasmon band. If the particles are incompletely passivated, then the absorption spectra may display a large shift of the plasmon resonance and characteristic increase in near infra-red absorption [323]. A representative figure comparing particle aggregation after dialysis is shown in Figure 7.3.

Gel Electrophoresis of Large Gold Nanoparticles

1. Cast a 0.5% 0.5X TBE agarose gel. Use a comb with smallest teeth available. The teeth of the comb used in this protocol are 3 mm x 1.5 mm x 10 mm.
2. Mix 15 µL gold nanoparticle sample with 5 µL 15% Ficoll solution as the loading buffer.
3. Load samples into the gel with 0.5X TBE as the running buffer. Reserve a lane in the gel for a DNA marker such as New England Biolab’s Tridye 1 kb DNA marker. The Tridye marker contains three organic dyes which are a good visualization tool to monitor the progress of the gel.
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Figure 7.3: The absorption spectrum of 80-nm gold nanoparticles passivated with a monothiol ligand (red squares) taken after dialysis shows increased near-infrared absorption with respect to the unpassivated particles before dialysis (black curve). The near-infrared absorption band is indicative of aggregation. The spectrum taken after dialysis of particles passivated with DHLA-SBE (green circles) do not show the same increase. All spectra are normalized to the absorption maximum at the plasmon resonance wavelength. Reprinted with permission. Copyright Springer.

Figure 7.4: The brightfield illuminated (A), UV illuminated (B), and overlaid images (C) of a 0.5% 0.5X TBE agarose ethidium bromide stained gel. Lane 1 is New England Biolab’s Tridyke 1kb DNA ladder while lanes 2 and 3 are the passivated and unpassivated particles, respectively. The passivated particles (red arrow) move with quickly into the gel while the unpassivated particles (black arrow) remain near the load well after 35 minutes at 6.5 V/cm. Reprinted with permission. Copyright Springer.
CHAPTER 7. LIGAND SYNTHESIS AND PASSIVATION FOR SILVER AND LARGE GOLD NANO PARTICLES

Figure 7.5: Incompletely passivated large gold nanoparticles will become immobilized at the top of various liquid chromatography resins. Here, unpassivated 80 nm diameter gold nanoparticles have become immobile on a Sephacryl S-300 column. Reprinted with permission. Copyright Springer.

4. Run samples at a field strength of 6.5 V/cm.

5. Record a brightfield image of the particle on a lightbox. The large gold nanoparticles, if properly passivated, should run as a tight pink band. A representative gel is seen in Figure 7.4.

Column Test for Large Gold Nanoparticles

1. Prepare 10 mL of a 50% slurry of Sephacryl S-300 size exclusion resin with 18.2 MΩ-cm water.

2. Pack a glass drip column (1.5 cm inner diameter) with 5 mL of slurry.

3. Equilibrate the column with two column volumes of water.

4. Let the water drip through column until the meniscus of the mobile phase reaches the top of the packed resin bed.

5. Once the water has reached the top of the column, carefully load 500 µL of passivated particle sample from Section 7.4 to the top of the column.

6. Let gold sample move into the column.

7. After the gold has entered the top of the resin bed, carefully wash the column with enough water to wash the particles through the column. Incompletely passivated gold nanoparticles will remain at the top of the column, such as in Figure 7.5.
8. Repeat column test with other anion exchange, cation exchange, DEAE, and hydroxyapatite resins from Bio-Rad’s chromatography media sampler pack to determine scope of usable resins with the particle system. The passivated particles should not aggregate at the tops of the columns.
Chapter 8

Conclusions and Future Directions

8.1 Status of 3DSPTS

In the past few years, the single particle tracking spectroscopy field has focused on proof-of-concept experiments despite the improvement in tracking and trapping systems. Current implementations are able to use fewer photons with better scanning techniques [324] or track smaller molecules, even down to the single molecule limit [130, 196, 325, 326]. The ability to hold a molecule or particle within the focus of a confocal microscope and perform spectroscopic measurements has been demonstrated [69, 116]. The challenge ahead is to ask questions which take advantages of the tracking system’s unique capabilities: extended temporal and spatial measurements with correlated spectroscopy.

One of the main bottlenecks to using single particle tracking spectroscopy is the robust conjugation of biological molecules to tracked inorganic probe particles. Endogenous and exogenous fluorescent biological molecules bleach too quickly to allow for long-time, spatially-correlated dynamical measurements in complex environments. This necessitates the use of photo-stable hierarchical nanoparticle systems. Ideal particle conjugation should not induce changes to biological dynamics, and be resistant to non-specific biomolecule absorption.

8.2 Challenges with Nano-Bio Interfaces

Large gold nanoparticles are useful tools because they are bright, photostable and have a known surface chemistry. The preparation of a conjugated nano-bio particle system requires, prevention of non-specific binding, a well controlled attachment-chemistry scheme, and also a purification scheme to enrich the percentage of designed conjugated particles.

Problems with Streptavidin-Biotin Conjugation

Streptavidin is an approximately 50 kDa tetrameric protein which binds biotin with a $K_D \approx 10^{-15} M$ [309]. It is one of the strongest non-covalent interactions known. It is widely
used in the biotechnology field for generating ‘pull-down’ type assays. Large polystyrene bead coated with streptavidin bind some biotinylated species such as DNA or a protein. The attached biotinylated species then interacts with some other biological molecule in a mixture of other molecules. The bead is then separated and the supernatant is washed away. Subsequent release of the interacting species can help identify new interactions. This assay is similar in spirit to DNA fingerprinting assays or western blot analysis which can separate interacting species via electrophoresis.

Diluted free streptavidin in the absence of biotin does not form a stable tetramer. Figure 8.1 shows the chromatogram of free streptavidin (3 mg/ml) on a Superdex 75 size-exclusion column. Larger species will elute off the column first because they are less able to enter the resin’s pores. Figure 8.1 compares the chromatogram streptavidin with and without pre-incubation with biotin. In the presence of biotin, streptavidin elutes off the column earlier, which is assigned to stabilization of the tetramer structure. Desthiobiotin [327], a biotin mimic with a lower $K_D$ ($\approx 10^{-13}$), can be displaced by biotin and also stabilize streptavidin.

80 nm gold nanoparticles were passivated with a biotinylated ligand, dialyzed, incubated with desthiobiotin-stabilized streptavidin, and then dialyzed a second time to remove unbound streptavidin. However, even after attachment of the biotin and incubation with stabilized streptavidin, the gold particles are still susceptible to aggregation, as seen in 8.2. In principle, any streptavidin bound to the gold should have remained in the tetrameric struc-
Figure 8.2: Tube A is a solution of 80 nm biotinylated gold nanoparticles. Tube B is solution of biotinylated 80 nm gold nanoparticles with excess desthiobiotin. Tube C is a solution of biotinylated 80 nm gold nanoparticles with streptavidin after addition of excess desthiobiotin before second round of dialysis. Tube D is a solution of biotinylated 80 nm gold nanoparticles with streptavidin after addition of excess desthiobiotin after second round of dialysis to remove excess desthiobiotin. Tube C was the same color as Tube B before addition of excess desthiobiotin. Aggregation of gold nanoparticles leads to decreased pink color.

As seen in Figure 8.2, incubation of the dialyzed streptavidin-coated gold nanoparticles causes the particles to aggregate. The dialyzed biotinylated-coated gold nanoparticles without streptavidin do not aggregate in the presence of streptavidin. General principles for how biological materials interact with nanoparticle surfaces are still needed.

**Generation of Monovalent Streptavidin**

In order to prevent aggregation of the streptavidin in the presence of biotinylated gold nanoparticles, streptavidin can be pre-stabilized with the addition of biotinylated DNA. Here, 60 µl 1.7 µg/µl 15bp of double stranded DNA are incubated with 30 µl 10 mg/ml streptavidin (2:1 DNA:streptavidin). The DNA streptavidin can be purified by anion exchange chromatography. Increasing numbers of DNA molecules require a greater percentage of NaCl in the mobile phase to elute off the column. The appearance of multiple peaks in the streptavidin-dna mixture (black curve Figure 8.4) and the absence of those peaks in the free streptavidin curve (inset Figure 8.4) are putatively assigned as discrete conjugates of streptavidin and DNA. Aliquots of double stranded DNA can be used to calibrate the
MonoQ anion exchange column used in this experiment.

Decreasing the slope of the NaCl salt gradient enables resolution between isolated DNA molecules of length 15, 30, 60 and 90 bp 8.4 (red curve Figure 8.4). This allows for the possibility of using the MonoQ column to resolve streptavidin molecules with controlled valency. A similar approach was developed by the Ting group using a step-wise $Ni^{2+}$ column to purify streptavidin molecules with known valency [328]. However, step-wise $Ni^{2+}$ gradients are not as well controlled because they causing leaching of all bound species off of the column, making separation more difficult.

Recovery of discrete conjugates of biotinylated DNA with streptavidin is complicated by low recovery of the collected fractions. However, this problem is not insurmountable and may be addressed in the future. Photo-cleavable biotin linkers are commercially available and can be used after purification from the anion exchange column to remove the DNA strands linked to streptavidin.

**Discrete 1-1 Particle Conjugation**

In principle, the long observation time granted by the single particle tracking spectroscopy technique allows one to look for multi-scale dynamical changes in a biological system. Towards this end, one needs to be able to assign changes in the spectral fluctuations of the tracked particle to dynamical changes from single biological molecules instead of from the collective fluctuations of a few biological molecules.

Discrete conjugates of biological molecules with small inorganic nanoparticles have been achieved in the literature for small gold nanoparticles and quantum dots [313]. Purification of these conjugated materials is straightforward using electrophoresis. For other particles such as large gold nanoparticles, the surface chemistry of the nanoparticles makes this challenging.
CHAPTER 8. CONCLUSIONS AND FUTURE DIRECTIONS

Figure 8.4: MonoQ anion exchange chromatogram of 15, 30, 60 bp dsDNA mixture (red) and streptavidin pre-incubated with biotinylated 15 bp dsDNA (black). Inset shows chromatogram of streptavidin in absence of DNA. Free streptavidin releases from the column at a relatively low conductance value. Pre-incubation with DNA causes multiple peaks to appear. Inset shows the free unconjugated streptavidin eluting from the column at much lower conductance values.

Ligands are more labile and the particles do not have as a great a tolerance to changes in buffer pH and salt concentration. General methods for passivating and conjugating large gold nanoparticles to biological materials has been described in this thesis. The goal of forming 1-1 discrete conjugates, although made closer by the work described in this thesis, is still illusive.

Several schemes were devised and attempted during the course of this thesis in order to try to tackle this issue. In one scheme, M13 phage DNA was annealed to a monothiolated 200 base single-stranded DNA molecule and then conjugated to gold nanoparticle surfaces. In another scheme, 2kb long DNA with thiolated primers was linked to large gold nanoparticles. In neither scheme was there any detectable change in mobility of the large gold nanoparticles as a result of attachment. Phage complementation and thiol-activity assays confirmed the sequences and thiol activity. It was found in this thesis work that in order to attach biological molecules to gold nanoparticles, one needs to have a $10^6$ fold excess of a DHLA-derived ligand. The concentrations of the phage DNA and thiolated PCR products may have been too low and too labile to have properly attached to the gold surface. Use of DHLA-linked DNA, described in chapter 7, may enable these schemes to work in the future and they should be revisited.
8.3 Future Directions

**DNA Looping Transcription Regulation**

One of the ways transcription is regulated is via DNA looping. One of the early examples comes from the NtrC system. NtrC is an enhancer binding protein which regulates the glutamine synthesis gene, important for conversion of glutamine and glutamic acid. The primary evidence for regulation of transcription by direct contact of the enhancer via a DNA loops comes from two papers which performed TEM and AFM analysis of the DNA with RNAP, sigma factor, and enhancer, bound in direct contact via a DNA loop [329, 330]. In both of those early papers, the DNA contained the promoter and enhancer binding sequences. However, they artificially extended the number of bases separating the two of them, thereby lowering the activation barrier to loop formation. The native sequence does not contain an IHF binding site, which is known to help bend DNA.

Models of DNA fluctuations, like the Zimm [331] and Rouse [332] models, are useful for modeling the persistence length of long DNA sequences such as in lambda-phage [333–335]. However, as the number of base pairs becomes low these models are insufficient to describe the behavior and underestimate the conformational flexibility of the short DNA strands. Single particle tracking spectroscopy may be able to provide new biological insights. One can now couple DNA to a tracked gold particle and measure shape fluctuations over time. As long as there is only one DNA attached, one could assign changes in shape to changes in looped state as well as watch the temporal assembly.

**In-Vivo Temperature Clamping**

Elevated temperature is associated with various pathologies, such as cancer [336, 337]. Intracellular temperature is heterogeneous. Yang et. al. used the temperature-dependent spectral shifting of CdSe/ZnS quantum dots [38, 88] to measure the local temperature at different points within single NIH/3T3 cells [22]. In their experiment, quantum dots were electroporated and showed temperature differences of up to 12°C. It is known that temperature can influence a variety of intracellular processes such as gene expression, intracellular transport, and protein folding.

Using nanoparticles, one can not only measure but also modulate biological processes using controlled targeted temperature stimulation. Huang et. al. used radio-wave heating of magnetic nanoparticles to stimulate the opening of an ion channel [338]. Optically induced hyperthermia of targeted gold nanoparticles are used as potential cancer therapeutic to kill diseased tissue [283].

Because nanoparticles can both measure and introduce temperature locally. Measuring and modulating heating opens the possibility of constructing a nanoparticle heterostructure which could ‘lock’ temperature locally using real-time closed-loop control. Targeting peptides can be robustly installed onto these particle constructs allowing for targeted closed-loop intracellular temperature control.
8.4 Conclusion

There are two main approaches to scientific research. The first is to use currently available
tools to answer hypothesis driven questions. The second is to develop new general tools
that can help answer questions which could not have been answered with more established
methods. To date, the work in this thesis has contributed part to the second approach in
order to study complex systems. The goal of 3D single particle tracking spectroscopy has
been to perform long-time or spatially-correlated spectroscopy in complex environments. It
takes time to develop a new technique robustly but recent developments should make one
optimistic about future opportunities in this field.
Bibliography

(21) Turing, A. M. Phil. Trans. R. Soc. 1952, 237, 37–72.


(201) To improve numerical stability in calculating the fluctuation correlation in spectral contrast, the following approximation is used,

\[ C_{\chi c}(t) \equiv \langle \delta \chi_c(t) \delta \chi_c(0) \rangle = \frac{\langle I_R(t) - I_B(t) \rangle \langle I_R(0) - I_B(0) \rangle}{\langle I_R(t) + I_B(t) \rangle \langle I_R(0) + I_B(0) \rangle} - \langle \chi_c(t) \rangle^2 \]


(204) Comparing the decay times of the same particle at different temperatures and viscosity can also verify the DSE relation, however, due to the particle’s size inhomogeneity of a bulk sample, it is impossible to find identical particles and thus it is much easier to keep the viscosity and temperature constant and compare the decay times of different particles.


(263) Werner, A Annalen Der Physik 1907, 24, 164–190.
(268) Palasz, A; Czekaj, P Acta Biochimica Polonica 2000, 47, 1107–1114.


Appendix A

Cloning NtrC Sequences into M13 Phage

NtrC Sequence - TCG ACG TTT AAA CAC GAG ATC TTC AAT TCT GCT GGC CGT GTC GCC GTA AAA ATA TAA AGC GAA ATC TGT GCC AAC TTT CAC ATT GCC CCC AAA AGG CGT TAT CAT GCG CAC CAT CGT GCA AAA GGG CTG CAC CAC GGT GAC TAT GTT GCA CCA AAA TAG TGC TTT AAT GTG AAC ATT AAG CAC CAC ATT GGG

NtrC Reverse Complementary - GAT CCC CAA TGT GGT GCT TAA TCA CAT TAA AGC ACT ATT TTG GTG CAA CAT AGT CAC CGT GGT GCA GCC CTT TTT CAC GAT GGT GCC CAT GAT AAC GCC TTT TGG GGG CAA TGT GAA AGT TGG CAC AGA TTT CGC TTT ATA TTT TTA CGG CGA CAC GGC CAG CAG AAT TGA AGA TCT CGT GTT GTC TAA ACG

Above sequences are reverse complementary with BamHI and SalI sticky ends for insertion into multiple cloning sequence of M13mp18 and M13mp19 phage. IDT DNA can synthesis above sequences because they are less than 200 bp length cutoff.

After dual BamHI and SalI digest of M13mp18, remove smaller digest fragment by agarose electrophoresis using standard gel and recovery protocols in a 0.5% 0.5 X TBE gel. Following recommended New England Biolabs protocol, annealed NtrC fragments into dual-digested M13mp18 with T4 DNA ligase. During first ligation, perform titration series of the amount of fragment to digested M13mp18. Too high excess of fragment can result in concatamers.

After ligation, use standard NEB transformation protocol to insert plasmid into NEB 5-alpha competent cells. NEB 5-alpha cells contain F-plasmid which permits phage propagation. Grow up over night to generate phage starter culture.
Appendix B

Shape Analysis via Rotational Correlation

Introduction

Information from a correlation of the spectral and polarization fluctuations carries more information than just the average tumbling time. The magnitude of the autocorrelation at zero time delay, \( \langle \chi_c(0)\chi_c(0) \rangle \), contains information about the shape of the particle.

Mathematical Relationship to Particle Shape

The following analysis follows the derivation given by Yang [124], where the scattering by the gold nanoparticle is modeled as a prolate spheroid.

\[
\langle \chi_d(t)\chi_d(0) \rangle = \frac{2}{15} \left( \frac{\alpha_{aniso}}{\alpha_{iso}} \right)^2 \left( \frac{3C + CH}{4A + 3B + BH} \right)^2 \left[ \sum_{n=-2}^{2} \left( D^{(2)}_{n,0}(\Omega_{DP}) \right)^2 e^{-6D_{\perp}t+n^2(D_{\parallel}-D_{\perp})t} \right]
\]
\[ D_{n,0}(\Omega_{DP}) = \sqrt{\frac{4\pi}{2l+1}} Y^*_{l,n}(\theta, \phi), \text{ where} \]

\[
A = \frac{1}{6} - \frac{1}{4} \cos(\Delta) + \frac{1}{12} \cos^3(\Delta)
\]

\[
B = \frac{1}{8} \cos(\Delta) - \frac{1}{8} \cos^3(\Delta)
\]

\[
C = \frac{7}{48} - \frac{1}{16} \cos(\Delta) - \frac{1}{16} \cos^2(\Delta) - \frac{1}{48} \cos^3(\Delta)
\]

\[ H = \cos(2\Theta_i) \]

\[ \Theta_i = \sin^{-1}(\text{NA}_{\text{cond}}/n_0) \]

\[ \Delta = \sin^{-1}(\text{NA}_{\text{obj}}/n_0) \]

\[ \alpha_{\text{aniso}} = (\alpha_3 - \alpha_1), \alpha_{\text{iso}} = \frac{1}{3} (\alpha_3 + 2\alpha_1) \]

\[ \alpha_3 \equiv a(1 + \xi), \alpha_1 \equiv a \]

\[ \xi = \frac{a_3}{a_1} - 1 \]

These equations simplify greatly because they reduce to functions of the numerical aperture of the condenser, \( \text{NA}_{\text{cond}} \), numerical aperture of the collection objective, \( \text{NA}_{\text{obj}} \), index of refraction, \( n_0 \), and shape parameter \( \xi \). Figure B.1 shows how \( \Theta_i, \Delta \) and \( \xi \) are defined. If one makes the reasonable assumption that the diffusion and polarization frames of the particle overlap, \( \Omega_{DP} = (0,0) \), then

\[ \sum_{n=-2}^{2} \left( D_{n,0}^{(2)}(\Omega_{DP}) \right)^2 e^{-6D \perp t + n^2(D \parallel - D \perp) t} = \frac{5}{4\pi} \text{ at } t = 0. \]

The simplified equation,

\[ \langle \chi_d(0) \chi_d(0) \rangle = \text{Constant} * f(\xi) \quad \text{(B.1)} \]

reduces to a function of just the shape parameter, \( \xi \).
Figure B.1: Schematic showing the relationship of the annular illumination of the condenser and objective. The shape parameter $\xi$ is defined in terms of a prolate spheroid encompassing an anisotropic gold nanoparticle.

Figure B.2: (a) Comparison of the normalized particle size distributions as measured by single particle tracking and TEM analysis. (b) Comparison of the normalized shape distributions as measured by single particle tracking and TEM analysis. Autocorrelation of polarization fluctuations enables the recovery of the particle sample’s size and shape distribution.
Figure B.3: TEM (blue) and 3DSPT (red) shape factors for 100 nm (73 particles), 150 nm (108 particles), and 200 nm (45 particles) particle samples are combined and histogrammed together. Shape distributions for both methods are qualitatively similar while the 3DSPT method underestimates the fraction of particles which show small degree of anisotropy (< 5%). Inset shows TEM of a 100 nm AuNP with fitted gaussian showing major and minor axes $a_3$ and $a_1$, respectively. $\xi = a_3/a_1 - 1$. 

\[ \xi = \frac{a_3}{a_1} - 1. \]