Image Deconvolution Techniques for Single Molecule Studies

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Image Deconvolution Techniques for Single Molecule Studies

by

Michael C. DeSantis

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requirements for the degree
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Abstract

One of the principal challenges in the field of biophysics, particularly that of protein-nucleic acid interactions, is the need to analyze information from single proteins as opposed to ensembles of many molecules. Consequently, the advent of high-resolution imaging in single molecule microscopy has enabled researchers to probe the underlying processes of gene regulatory networks and other biological systems. There is, nonetheless, a tradeoff between spatial and temporal resolution, or the ability to localize a molecule in space at increasingly shorter time scales. As such, this dissertation addresses these challenges that hinder single molecule studies by: (i) developing deconvolution techniques in order to localize both immobile and dynamic molecules from their single images with improved spatial and temporal resolution, (ii) determining a protein’s diffusive properties with high temporal resolution, and (iii) applying our analytical methods to study model biological systems.
Acknowledgements

First and foremost, the research presented herein was performed at Washington University in St. Louis in the biophysics laboratory of Dr. Yan Mei Wang, the principal investigator (and my thesis advisor) for whom I owe a great debt of gratitude to. During my tenure with the research group, she was able to offer guidance with many aspects of the various projects we were investigating while maintaining a clear focus and direction for future work. I would also like to acknowledge the additional members of my dissertation committee including Dr. Anders E. Carlsson, Dr. Zohar Nussinov, Dr. Ralf Wessel, Dr. Robert E. Blankenship, and Dr. Elliot L. Elson for providing feedback and encouragement for this work with particular appreciation to the former who has served on my progress report committee and has been an invaluable source for advice and assistance.

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instrumental in perfecting the experimental setup. Additionally, I would like to thank Jonathan M. Kessler and Anthony Kovacs who are continuing to work tirelessly on the latter two projects discussed in Chap. 4.

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*For Mom and Dad*
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Preface

This dissertation presents research that was conducted from Summer 2008 through Spring 2012 in Professor Y. M. Wang’s biophysics laboratory in the Department of Physics at Washington University in St. Louis. The nature of the work concerns the study of biomolecules using single molecule fluorescence imaging methods; specifically, developing analytical deconvolution techniques to extract information pertaining to the localization and differentiation of multiple fluorophores (Chap. 2) as well as the determination of protein diffusion coefficients from single images (Chap. 3) constituting an improvement in temporal resolution over existing methods. All of this work has been published to date with specific portions excerpted and/or modified for use in said chapters.

In my final year of study, I began investigations of several biological systems of interest (Chap. 4). Such collaborative projects have strengthened both the merit of the work and my aptitudes in theory as well as experiment. Furthermore, throughout my tenure in the group, I was responsible for writing a significant amount of code such as simulations of diffusion trajectories and their corresponding intensity profiles which were subsequently fit by Gaussian functions. This dissertation comprises five
chapters: the aforementioned ones, an introduction, and conclusion, as well as four appendices that serve as supplementary material which are referenced as needed.
Chapter 1

Introduction

The obvious inability of present-day physics and chemistry to account for [the events in space and time which take place within the spatial boundary of a living organism] is no reason at all for doubting that they can be accounted for by those sciences.

- Erwin Schrödinger, *What is Life?* (1944)

1.1 Single molecule studies

Molecular biophysics is a rapidly expanding interdisciplinary research area that encompasses many fields including molecular biology, biochemistry, and virology with the goal of addressing various biological questions of interest using quantitative methods. Both technical and theoretical challenges in meeting these demands have prompted the development of new imaging techniques as well as experimental approaches capable of manipulating biological systems. The advent of single molecule imaging via fluorescence microscopy has enabled researchers to extract information pertaining
to the localization and dynamics of individual molecules not accessible from bulk, ensembled-averaged measurements [1–4]. Although the single molecule field is relatively young, with its emergence in 1989 documented by the detection of the first single molecule observed in crystal at liquid-helium temperatures by Moerner and Kador, it is continuing to grow exponentially [5, 6].

Previous studies could only measure the collective behavior of many molecules, from millions to Avogadro’s number \(6.02 \times 10^{23}\); however, single molecule experiments can truly explore the regime in which an individual protein samples and interacts with its local environment permitting us to probe the underlying, complex biological processes that occur, for example, in a living cell with \textit{in vivo} imaging studies as first achieved more than ten years ago [7, 8]. Figure 1.1 illustrates a simple hierarchy of biological systems from a typical mammalian cell to an individual protein to better appreciate the spatial scales involved.

Figure 1.1: Hierarchy of biological systems on a logarithmic scale. Cellular processes occur over a broad spatial range and are often mediated by simple molecules that interact with others in increasingly complex systems such as a typical mammalian cell that is 10 \(\mu\)m in size. To investigate these processes, conventional optical microscopy can be employed for \textit{in vivo} studies while single molecule fluorescence imaging methods report measurements on the order of nanometers permitting \textit{in vitro} experiments as well with improved spatial resolution.
1.1 Single molecule studies

Depending on the biological system being studied and the information needed, multiple single molecule methods are available, which are mainly classified as (i) force-based manipulation and detection such as optical tweezers and atomic force microscopy (AFM) [9], and (ii) fluorescence imaging and spectroscopy including total internal reflection fluorescence (TIRF) [10], confocal, and two-photon excitation microscopy. These latter techniques permit fluorescence correlation spectroscopy (FCS) [11–13] and fluorescence recovery after photobleaching (FRAP) [14], which are both commonly used to ascertain a molecule’s diffusion coefficient as well as Förster resonance energy transfer (FRET) [15–18], which is capable of measuring distances between chromophores separated less than $\sim 10$ nm.

Fluorescence microscopy takes advantage of the unique properties of fluorophores such as the green fluorescent protein (GFP) depicted in Fig. 1.1. Whereas GFP is an organic fluorophore such that cell lines can be genetically modified to express this protein as a fusion construct, synthetic dyes and quantum dots can also be used to label proteins of interest that are not naturally fluorescent. Imaging is accomplished noninvasively with minimal perturbation to the system being studied due to the probes’ relative small size, thereby permitting observation of the dynamic processes occurring inside the cell. Upon stimulation with laser light, typically in the visible electromagnetic spectrum, a fluorophore undergoes a transition to an excited state. Following energy loss associated with Stokes shift, the molecule relaxes to its ground state simultaneously emitting photons of a longer wavelength during the process. These photons can be detected by various means, however, in order to visu-
alize and ultimately localize the corresponding molecules from their emissions, which appear in the microscope as the diffraction-limited spots shown in Fig. 1.7 A, an electron multiplying charge coupled device (EMCCD) camera is required. Each pixelated frame from a movie represents the total number of photons detected within the given exposure time or how long the molecules are excited for. To minimize the contribution from background fluorescence thereby improving the signal-to-noise ratio (SNR) for images of such sparse, punctate objects, we employ TIRF microscopy for our experimental studies.

1.1.1 TIRF microscopy

Developed in the early 1980s by D. Axelrod at the University of Michigan, TIRF uses an ‘evanescent wave’ to selectively excite fluorophores in an aqueous environment near the glass-water interface [10]. For light, initially traveling in a solid (e.g., glass) and incident at a high angle $\theta$, upon the glass-water interface, the condition for total internal reflection (TIR) is in accordance with Snell’s law such that if the refracted light travels completely parallel to the surface then the critical angle is given by

$$\theta_c = \arcsin \left( \frac{n_2}{n_1} \right),$$

(1.1)

where $n_1$ and $n_2$ are the indices of refraction for the solid and liquid, respectively, assuming $n_1 > n_2$. This process is illustrated in Fig. 1.2 A for $\theta > \theta_c$ in which all of the light is reflected back; conversely, for $\theta < \theta_c$, some of the light refracts through
1.1 Single molecule studies

at an angle measured with respect to the normal.

If the conditions for TIR are met such that the reflected and incident light are of the same amplitude, Maxwell’s equations in a dielectric medium for the given boundaries predict a non-vanishing solution for a transmitted wave at the surface. Using a Gaussian beam, the resulting evanescent wave must have an intensity that decays exponentially as a function of the perpendicular distance \( z \), from the surface as

\[
I(z) = I_0 \exp\left(\frac{-z}{z_d}\right),
\]

and attenuates until the characteristic penetration depth

\[
z_d = \frac{\lambda}{4\pi \sqrt{n_1^2 \sin^2(\theta) - n_2^2}},
\]

typically less than 200 nm where \( \lambda \) is the wavelength of the incident light in vacuum.

Figure 1.2 B shows a schematic for the microscope-imaging system with a specimen prepared from a 5 \( \mu \)L aliquot of solution (\( n_2 = 1.33 \)) containing water (buffer) and fluorescent proteins sandwiched between a fused-silica chip (\( n_1 = 1.46 \)) and a glass coverslip, resulting in the creation of a 10 \( \mu \)L thick water layer. A linearly polarized laser line (see Fig. 1.3) was focused by a quartz prism (using an immersion oil with a comparable index of refraction and which serves as an optical connection) to a 40 \( \times \) 20 \( \mu m^2 \) region of the fused-silica surface. The corresponding penetration depth \( z_d \), for \( \lambda = 488 \) nm (the spectral absorption peak of GFP) was calculated to be 117
1.1 Single molecule studies

Figure 1.2: Schematics for the microscope-imaging system with a prism-type TIRF setup. (A) Under TIR conditions for $n_1 > n_2$, when the excitation light’s angle of incidence is greater than the critical angle ($\theta > \theta_c$), an evanescent wave propagates at the glass-water interface exciting nearby fluorescent molecules. The penetration depth is typically less than 200 nm. (B) A 5 µL aliquot of solution is sandwiched between a fused-silica chip and a glass coverslip. Using prism-type TIRF, photons emitted by fluorophores under illumination are detected by an EMCCD camera imaged through an inverted microscope.
nm constituting a small fraction of our water layer such that only fluorophores near the glass-water interface are illuminated. Consequently, fluorophores freely diffusing in the bulk media are not excited and do not contribute to the background noise. Figures 1.4, A and B show the laboratory’s microscope-imaging system and laser light incident on a specimen under TIRF conditions, respectively.

1.2 Diffraction-limited system

A microscope’s objective as opposed to any other component is critical for extracting information from any obtained images. Its performance limits the field size that can be used, an image’s contrast, and our ability to resolve particular details within it [19]. For over 170 years, optical microscopy has been able to shed light on the cell and other systems of interest leading to the development of modern molecular biology and its biomedical applications. Many of these experiments are conducted in the Fraunhofer or far-field diffraction regime whereby the pattern or image, formed on an observation screen by plane waves emitted from a distant point source diffracted by an aperture, only changes in size and not its shape as the screen is moved farther away. The condition for Fraunhofer diffraction occurs when \( R > \frac{w^2}{\lambda} \), where \( R \) is the smaller of the two distances from either the point source to the aperture or from the aperture to the observation screen, \( \lambda \) is the wavelength of the emitted light, and \( w \) is the width of the aperture [20]. To achieve optimal performance, high photon efficiency is required such that sacrificing the useable field size and chromatic correction for
1.2 Diffraction-limited system

Figure 1.3: Schematic for the TIRF and epifluorescence optical setup of the laboratory using an Argon/Krypton laser with multiline output. The laser ends at the fluorescence microscope attached to an EMCCD camera.
Figure 1.4: Images of the laboratory’s microscope-imaging system under prism-type TIRF conditions. (A) Our Nikon Eclipse TE2000-S inverted microscope (Nikon, Melville, NY) attached to an iXon back-illuminated EMCCD camera (DV897ECS-BV; Andor Technology, Belfast, Northern Ireland). (B) A 488 nm laser line incident on the specimen held by the microscope stage under prism-type TIRF conditions. The quartz prism is illuminated with blue light that proceeds to undergo TIR for our angle of incidence.
greater transmittance with fewer lens elements is often favored. Additionally, the use of high numerical aperture (NA) oil-immersion objectives with the NA reaching about 1.5 in modern optics, allow for greater resolving power with minimal loss due to spherical aberration effects.

Nevertheless, biochemical and DNA-based techniques were and continue to be used for investigating many cellular processes not accessible by conventional far-field optical microscopy since the amount of information that can be ascertained by the latter is restricted by the diffraction limit of the system. The Abbe limit in the lateral dimensions, derived in 1873, was postulated to be due to the wave nature of light and therefore incapable of being circumvented,

\[ \Delta l_{\text{Abbe}} = \frac{\lambda}{2n \sin(\theta)} = \frac{\lambda}{2\text{NA}}, \quad (1.4) \]

where \( n \) is the index of refraction that light with wavelength \( \lambda \), is traveling in, and \( \sin(\theta) \) is the half angle over which the objective gathers light from the sample. A system is considered diffraction-limited if the resolving power is limited solely by the instrument’s optics and usually achieved when the width of the point spread function (PSF), or the system’s response to a point light source, is comparable to the Abbe limit [Eq. (1.4)].
1.2.1 PSF and the resolution of imaging systems

A three-dimensional PSF is generated from a point light source when imaged with a diffraction-limited system. Plane waves impinging on an opaque screen containing a circular aperture, preferable to a rectangular one, produces a diffraction pattern, specifically the Airy pattern as illustrated in Fig. 1.5 A, with axial symmetry such that a cross-section perpendicular to this optical axis is shown in Fig. 1.5 B. It is marked by a central maximum or circular spot with high intensity denoted as the Airy disk surrounded by a dark ring with secondary maxima radiating outwards. Mathematically, the Airy pattern has an irradiance or intensity that is dependent on the angle of observation, $\theta$, and given by

$$ I(\theta) = I_0 \left[ \frac{2J_1(ka \sin(\theta))}{ka \sin(\theta)} \right]^2, \quad (1.5) $$

where $I_0$ is the maximum intensity of the Airy disk, $k$ is the wavenumber equal to $2\pi/\lambda$, $a$ is the radius of the circular aperture, and $J_1(u)$ is the Bessel function of the first kind of first order.

In single molecule fluorescence microscopy, we image multiple objects at a time with each fluorophore treated as an incoherent point light source. The image that is formed of the extended object sometimes consists of partially overlapping PSFs due to the proximity of the fluorophores; in general, for a larger object that is self-luminous, it can be regarded as an array of incoherent point sources with the resulting image appearing as a diffuse, diffraction-limited spot representing the instrument's
maximum achievable resolution [20]. Imaging via a circular aperture, where the PSF is described by an Airy pattern, the corresponding limit to the system’s resolution in the lateral dimensions was refined in 1896 as the Rayleigh criterion

$$\Delta l_{Rayleigh} = \frac{1.22\lambda}{2NA},$$  \hspace{1cm} (1.6)

determined according to the first nontrivial zero of $J_1(u)$. Qualitatively, it is the distance where the first minimum of the Airy function occurs (i.e., the edge of the Airy disk or first dark ring) such that if another incoherent point source of equal irradiance were placed at this position, it defines the threshold for being able to distinguish the two objects within the same diffraction-limited spot. This principle is illustrated in Fig. 1.6 for the cases in which two point sources are separated less than and greater than the Rayleigh criterion. For GFP with an emission peak of $\lambda = 509$ nm and a microscope objective with $NA = 1.49$, the resolution limit of our imaging system is $\approx 210$ nm.
1.2 Diffraction-limited system

Figure 1.6: Illustration of the Rayleigh criterion. The minimum details that can be resolved in an image occurs at the Rayleigh criterion (center), or the distance between two incoherent point sources of equal irradiance such that the central maximum of one image coincides with the first minimum of the other. If the distance is greater than the Rayleigh criterion (left), the corresponding Airy patterns are well separated such that the two objects are resolvable and if the distance is smaller (right), the corresponding Airy patterns overlap such that the two objects are not distinguishable within the same diffraction-limited spot. Image taken from http://hyperphysics.phy-astr.gsu.edu/hbase/phyopt/raylei.html.

1.2.2 Gaussian approximation of the PSF

In order to extract information pertaining to the molecule’s position within the diffraction-limited spot as well as the size of the spot itself, several algorithms are available. In contrast to the centroid method which is based on averaging over the pixel coordinates along a given axis and weighting them accordingly by the pixels’ intensity values, a direct Gaussian fit to the image remains unbiased and also represents a maximum-likelihood estimate when fitting via least-squares minimization assuming an underlying Gaussian probability distribution for the native PSF [21, 22]. Although the PSF is actually described by an Airy pattern with a central peak that drops off rapidly but with an overall irradiance that decays slowly to zero as the radial distance increases, it would be impossible to measure the root mean square (rms)
1.2 Diffraction-limited system

spot size directly as it is undefined. Ignoring the outer rings and approximating only the central lobe, comprising 84% of the integrated intensity, by a Gaussian because it is more mathematically tractable permits a simple determination of the fluorophore’s probable location such that the differences are minor in practice [20, 23]. The method has become widely adopted in single particle tracking (SPT) studies [24].

Figure 1.7: Image acquisition and 2D Gaussian fitting to the PSF. (A) Frame image of multiple GFP molecules primarily immobilized on a fused-silica surface appearing as diffraction-limited spots acquired using an EMCCD camera; the dim spots are due to molecules freely diffusing in the background during the frame’s exposure time. The scale bar is 2 µm. (B) Single pixelated image depicting the intensity profile for one of the molecules in A. The scale bar is 400 nm. (C) Corresponding 2D Gaussian fit to the intensity profile in B from which the molecule’s probable location in the lateral plane and SDs are determined.

The intensity profile or impulse-response function (equated to the PSF for the case of a stationary or immobile molecule) of a single image, such as the one shown in Fig. 1.7 B, is fitted to the following 2D Gaussian function utilizing the least squares curve fitting algorithm (lsqcurvefit) in the optimization toolbox provided by MATLAB (The Mathworks, Natick, MA) that solves nonlinear problems in a least
1.2 Diffraction-limited system

squares sense:

\[ f(x, y) = f_0 \exp \left[ -\frac{(x - x_0)^2}{2s_x^2} - \frac{(y - y_0)^2}{2s_y^2} \right] + \langle b \rangle, \]  

(1.7)

where \( f_0 \) is the amplitude, \( s_x \) and \( s_y \) are the standard deviations in the \( x \) and \( y \) directions, respectively, \( x_0 \) and \( y_0 \) are the centroid location of the molecule in the lateral plane, and \( \langle b \rangle \) is the mean background offset (see Figs. 1.7 C and 1.8). Determining the parameters require starting values ideally near their actual ones to avoid potential local minima that may hinder or prevent the optimization from proceeding; placing bounds on the parameters helps to address this concern. For each of the fitting parameters, there is an associated probability or likelihood function. The set of optimal values are determined from maximizing the likelihood by minimizing the explained sum of squares or residuals simultaneously with respect to each of the \( m \) parameters. This yields \( m \) coupled, partial differential equations such that multiple algorithms are capable of searching this \( m \) dimensional space with a variable step size to achieve faster convergence [25].
1.3 Single molecule localization with nanometer precision

The Rayleigh criterion dictates that for a widefield microscope using visible light, the maximum resolution that can be achieved is $\sim 210$ nm. In order to circumvent this threshold, a new class of ‘super-resolution’ techniques has emerged including those that extract information from the excitation and emission intensities such as 4Pi, spectrally selective imaging, and structured illumination microscopy [26–28], as well as those that require experimental modifications that take advantage of the probe’s fluorescence characteristics to perform image reconstructions. These latter ‘functional’ methods can be further classified as either deterministic in that fluorophores’ nonlinear response to excitation can be exploited including STED [29] and SSIM, or stochastic in that fluorophores can be selectively activated due to their photo-switchable behavior including STORM, PALM, and fPALM [30–32]. Although these methods provide a means to localize a single molecule within a small region of interest without additional fluorescence from neighboring sources, stochastic super-resolution techniques require complementary ‘super-localization’ analysis by fitting each image to a Gaussian function. Single molecule localization is typically accomplished by measuring the variance to a distribution of successive centroid measurements from repeated excitations (i.e., image stack).

Alternatively, localization and its associated error can be measured given just a single image of the molecule’s intensity profile. This is particularly useful since
fluorescent molecules are prone to photobleaching, the irreversible decomposition into the excited state because of their interaction with molecular oxygen before emission; consequently, localization errors may not be calculable for an insufficient number of images due to either photobleaching or if the molecule diffuses beyond the imaging region as is the case for dynamic systems. The technique is premised on the fact that although an object’s size is limited by the instrument’s resolution, its centroid can be determined with arbitrary precision according to the number of photons $N$, detected during the image’s exposure time. An analytical expression for the centroid error was derived by Thompson, Larson, and Webb [2] as

$$\Delta x_{rms} = \sqrt{\frac{s^2 + \frac{a^2}{12}}{N} + \frac{8\pi s^4 \sigma_b^2}{a^2 N^2}},$$

(1.8)

where $s$ is the standard deviation (SD) from a Gaussian fit to the intensity profile, $a$ is the camera’s pixel size, and $\sigma_b^2$ is the variance of the background noise. It is evident that the error due to shot noise, which scales as $1/\sqrt{N}$, usually dominates; therefore, efficient emitters with high quantum yields are desired for achieving nanometer precision as demonstrated by Yildiz et al. for localization measurements of myosin V walking on actin [3].

While Gaussian fitting provides a simple and convenient method for determining a molecule’s probable location in the lateral plane, the SD is measured simultaneously but often neglected. This quantity, first introduced by Pearson in his 1894 mathematical study of evolution [33] and also referred to as the width and spot size
SIMA and SMID studies

One of the principal challenges in the field of protein-nucleic acid interactions is the need to analyze information from single proteins or complexes as opposed to ensembles of many molecules. Consequently, the advent of high-resolution imaging in single molecule microscopy has enabled researchers to probe the underlying processes related to gene regulatory networks and similar biological systems. There is, nonetheless, a tradeoff between spatial and temporal resolution or the ability to localize a molecule...
in space at increasingly shorter time scales. As such, the research I am conducting, which culminates with this dissertation is dedicated to addressing these challenges which hinder single molecule studies; it relies heavily on SIMA to extract information pertaining to a protein’s localization and diffusive properties from individual images. My research is aimed at the following scientific objectives:

a. To develop SMID techniques in order to localize both immobile and dynamic molecules with improved spatial and temporal resolution. Attempts to circumvent the Rayleigh criterion ($\approx 210 \text{ nm}$) have yielded super-resolution methods that are capable of determining a particle’s lateral position within several nanometers by fitting the molecule’s observed intensity profile with a Gaussian function. A theoretical derivation of this localization error given a single image has been performed and modified to account for various artifacts single molecule imaging is subject to. Moreover, we have deduced an even smaller error associated with the width or spatial extent of the molecule’s intensity profile, which is used to infer its axial position as well as measuring the separations between multiple fluorophores within a diffraction-limited spot and characterizing an imaging system’s resolution. Subsequently, we can perform 3D localization analysis of molecules on the timescale of milliseconds or shorter constituting an improvement over current imaging modalities that require observation times nearly two orders of magnitude longer. The improvement in temporal resolution is made without sacrificing spatial resolution and permits the study of dynamic systems.
b. To determine a protein’s diffusive properties with high temporal resolution. How proteins interact with their environment is important for elucidating the dynamics of various biological systems. Among the current methods for determining protein diffusion coefficients such as FCS, which uses autocorrelation analysis on the fluctuation of fluorescence intensities within a measurement volume and FRAP, which measures the time for a previously photobleached spot to recover half of its initial integrated intensity by the diffusion of fluorescent molecules according to Brownian motion, only SPT preserves spatial information (i.e., localizations). Building on the deconvolution techniques, above, we are able to calculate a protein’s diffusion coefficient from single-image measurements (≤1 ms) used to determine its location in three dimensions and which demonstrates that fast tracking studies can be performed without loss in precision.

c. To apply the analytical tools we have developed to study model biological systems such as protein diffusion along DNA. According to the facilitated diffusion model, proteins locate their target sites on DNA by several diffusive mechanisms including one-dimensional sliding and three-dimensional random walks or hops. Currently, single molecule studies lack the temporal resolution required to distinguish one motion from the other. We have performed Monte Carlo simulations examining the role hops play in typical trajectories in order to calculate the respective diffusion coefficients from previous single molecule experiments. Moreover, the simulations suggest the proper time scales future
1.4 SIMA and SMID studies

studies need to assess hopping (and sliding) kinetics.

Additionally, we are conducting single molecule investigations using TIRF microscopy of two other biological processes including energy transfer in the phycobilisome (PBS), the light harvesting complex in cyanobacteria, as well as entry mechanisms of membrane signaling proteins undergoing intraflagellar transport (IFT) in *Chlamydomonas*. Initial observations indicate that light is emitted variedly throughout the PBS contrary to the belief that it occurs solely at the terminal pigments with high quantum efficiency. This is further supported by evidence revealing the presence of discrete ‘quenching subunits’, each comprising a group of fluorophores, which can be localized using SIMA methods. In *Chlamydomonas*, IFT is known to play a role in motility and signal transduction; however, this system is not well understood particularly with regards to how proteins enter the flagellum at its base. From movies obtained by *in vivo* imaging, SPT is performed in combination with Gaussian fitting. The diffusive process, as predicted according to two competing models, can be elucidated as well as the proteins’ diffusion coefficients from mean square displacement (MSD) analysis of their individual trajectories. Suggestive, albeit preliminary, results concerning anomalous diffusion within the entry region, distinct from IFT, are presented.
Chapter 2

Single molecule image deconvolution techniques

*Mathematics is the queen of the sciences.* -Carl Friedrich Gauss, from *Wolfgang Sartorius von Waltershausen, Gauss zum Gedächtniss* (1856)

We introduce single molecule image deconvolution methods for localization and tracking studies with improved temporal and spatial resolution over existing techniques. While measurements from repeated excitations of a molecule’s position in the lateral plane (*i.e.*, centroid) are traditionally required for tracking and determining its localization error, standard deviation measurements from Gaussian fitting to a single image can offer insight into the dynamics of single molecules at shorter time scales with greater precision. Furthermore, the standard deviation is often used to infer a molecule’s position along the axial dimension and can be used to measure the separa-
2.1 Precision analysis of standard deviation measurements

2.1.1 Introduction

Single particle tracking techniques can localize a molecule to well below the diffraction-limit [35–38] typically by fitting its respective intensity profile to a Gaussian function thereby determining its centroid. Although these methods should be accurate since Gaussian fitting represents a maximum-likelihood estimate, the precision associated with centroid measurements is used for further validation as well as providing a means of characterizing the achievable resolution. Any reported physical measurement including an object’s centroid and standard deviation (SD), which is used to infer its position along the axial dimension, must therefore have an associated error to it. While error analysis of single molecule point spread function (PSF) centroid measurements

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has provided the precision for localization measurements along the lateral dimensions
[2], and which enabled differentiation of various biological processes (such as the walk-
ing mechanism of myosin V on actin [3]), PSF SD measurement error analysis can
provide the precision in the following applications: (i) axial position measurements,
such that the SD of a single molecule’s PSF increases with the defocusing distance
[27, 30, 36]; (ii) single molecule orientation and polarization measurements, such that
a molecule exhibits an elliptical-shaped Gaussian PSF with SDs that change along
both lateral directions depending on its orientation with respect to the imaging plane
[39–41]; and (iii) characterization of an imaging system as being diffraction-limited
[42].

While the precision to centroid measurements has been investigated and subse-
quently verified from studying many systems [2, 3, 43], the precision of SD measure-
ments of single molecule PSFs has not. Our formulation is based on the fact that
although the size of a diffraction-limited spot is limited by the instrument’s resolution,
the object’s width or SD, like its centroid, can be determined with arbitrary precision,
principally dictated by the finite number of photons that are detected, \( N \) [2, 44]. Ad-
ditionally, the error is dependent on the standard deviation of the background noise
\( \sigma_b \), and the camera’s finite pixel size \( a \). Here, we report an analytical expression
for the error associated in measuring the SD of a molecule’s PSF as a function of
these parameters and compare it with the results following numerical integrations,
simulations, and experimental measurements achieving nanometer resolution.
2.1.2 Theory

Following the method developed by Bobroff [44] and subsequently used for centroid error analysis by Thompson, Larson, and Webb [2], we derive the error associated with SD measurements of single fluorophores, $\Delta s$. The approach uses Chi-square statistics to estimate the error when fitting experimental data to expected theoretical values. For consistency with previous single particle tracking (SPT) studies, we will retain many of the same notations used in Ref. [2]. Below, we derive the analytical solution for the SD error as a function of $N$, $a$, and $\sigma_b$ ($b$ in prior studies) beginning with one dimension and extending to two dimensions.

In 1D least squares fitting of the intensity profile of an immobile single fluorophore, $\chi^2(s)$ is proportional to the sum of squared errors between the observed photon count at pixel $i$, $y_i$, and the expected photon count $N_i(x,s)$, of a PSF. Here $x$ and $s$ are the measured position and SD of the PSF, respectively, while $x_0$ and $s_0$ are the true location and the theoretical SD of the molecule:

$$
\chi^2(s) = \sum_i \frac{(y_i - N_i)^2}{\sigma_{i,\text{photon}}^2},
$$

where $\sigma_{i,\text{photon}}$ is the uncertainty in the expected photon count at pixel $i$ without accounting for photon-to-camera count conversion (described in Appendix A.1). For simplicity, $N_i(x_0, s)$ is denoted as $N_i$ unless otherwise specified.

There are two sources for $\sigma_{i,\text{photon}}$ at pixel $i$: (i) the Poisson-distributed photon shot noise of the PSF where the variance is the mean expected photon count at the
2.1 Precision analysis of standard deviation measurements

pixel, \( N_i \), and (ii) the SD of the background noise, \( \sigma_b \), expressed in photons. The variances of the two sources add to yield

\[
\sigma_{i,\text{photon}}^2 = N_i + \sigma_b^2.
\]  

(2.2)

The deviation of \( s \) from \( s_0 \), \( \Delta s = s - s_0 \), is obtained by setting \( d\chi^2(s)/ds \) to 0, expanding \( N_i \) about \( s_0 \), and keeping the first order term in \( \Delta s \). Appendix A.2 shows the detailed derivation of \( \Delta s \) from \( d\chi^2(s)/ds = 0 \). The mean squared value of \( \Delta s \) is

\[
\langle (\Delta s)^2 \rangle = \frac{1}{\sum_i (N_i^2/\sigma_{i,\text{photon}}^2)} \Rightarrow \frac{1}{\sum_i (N_i^2/\sigma_i^2)},
\]  

(2.3)

taking photon-to-camera count conversion into consideration \( [\sigma_i^2 = \sigma_{i,\text{photon}}^2/M^2 = 2N_i + \sigma_b^2 + \langle b \rangle] \), where \( M \) is the photon multiplication of an electron multiplying charge coupled device (EMCCD) camera; see Appendix A.1.

To evaluate Eq. (2.3) in 1D, we use a normalized Gaussian distribution

\[
N_i = \frac{Na}{\sqrt{2\pi}s} \exp \left[ -\left( \frac{ia}{2s} \right)^2 \right],
\]  

(2.4)

where we set the location of the PSF to be at \( x_0 = 0 \) for simplicity and without loss in generality. We approximate the pixel summation in Eq. (2.3) by an integral going from negative to positive infinity, and we estimate \( \langle (\Delta s)^2 \rangle \) at the two extrema of \( \sigma_i^2 \): the high photon count regime where \( \sigma_b^2 + \langle b \rangle \) can be neglected, and the high background noise regime where \( 2N_i \) can be neglected. In the high photon count regime,
or shot noise-limited case, the error associated with the average SD measurement of a
collection of detected photons is given by the standard error of the standard deviation
\[ s_0 / \sqrt{2N(1 - 4N - 8N^2/3 - ...)} \] \cite{45-49}, which after accounting for photon-to-camera
count conversion yields a variance of

\[ \langle (\Delta s)^2 \rangle \approx s_0^2 / N, \] \hfill (2.5)

while in the high background noise regime,

\[ \langle (\Delta s)^2 \rangle = \frac{8\sqrt{\pi} s_0^3 (\sigma_b^2 + \langle b \rangle)}{3aN^2}. \] \hfill (2.6)

Although the summation in Eq. (2.3) assumed an infinitesimal pixel size, we know
that the measured SD of a photon should depend on it. Each photon is therefore
associated with two variances with respect to the centroid: (i) the mean variance of
the PSF, \( s_0^2 \), and (ii) a variance due to the fact that each photon is further binned into
a pixel which can be described as a uniform distribution with a width corresponding
to the pixel size \( a \) and a variance of \( a^2/12 \). Thus, the total variance of a photon due
to pixelation is their sum,

\[ s^2 = s_0^2 + \frac{a^2}{12}. \] \hfill (2.7)

Under experimental conditions, the measured s should be \( \sqrt{s_0^2 + a^2/12} \) and for the-
toretical formulations the expected SD of a PSF should include the pixelation effect.
2.1 Precision analysis of standard deviation measurements

We have verified that \( s^2 \) increases with \( a^2 \) according to Eq. (2.7) by simulation.

Given the expressions for both the high photon count and high background noise regimes, Eqs. 2.5 and 2.6 can be combined, while incorporating the pixelation effect added in quadrature, to obtain the 1D root mean square (rms) error \( \sqrt{\langle (\Delta s)^2 \rangle} \), or

\[
\Delta s_{\text{rms}} = \sqrt{\frac{s_0^2 + \frac{a^2}{12}}{N} + \frac{8\sqrt{\pi} (s_0^2 + \frac{a^2}{12})^{3/2} (\sigma_b^2 + \langle b \rangle)}{3aN^2}}.
\] (2.8)

Extending the calculation to 2D where \( s_i \) shall represent the SD in either the \( x \) or \( y \) direction of the imaging plane, and \( s_{0x} \) and \( s_{0y} \) are the theoretical SD values in the \( x \) and \( y \) directions, respectively, the SD error measured along the \( x \) dimension is

\[
\Delta s_{x,\text{rms}} = \sqrt{\frac{s_{0x}^2 + \frac{a^2}{12}}{N} + \frac{16\pi (s_{0x}^2 + \frac{a^2}{12})^{3/2} (s_{0y}^2 + \frac{a^2}{12})^{1/2} (\sigma_b^2 + \langle b \rangle)}{3a^2N^2}}.
\] (2.9)

The derivation of Eq. (2.9) is provided in Appendix A.3.

A more accurate estimation of \( \Delta s_{i,\text{rms}} \) can be obtained by numerically integrating Eq. (2.3), incorporating the transition region between the high photon count and the high background noise regimes. The numerical integration results are shown in Fig. 2.2 to be consistently higher than the analytical calculations by \( \approx 15\% \).
2.1 Precision analysis of standard deviation measurements

2.1.3 Methods

2.1.3.1 Sample preparation and imaging

Single molecule imaging was performed using a Nikon Eclipse TE2000-S inverted microscope (Nikon, Melville, NY) attached to an iXon back-illuminated EMCCD camera (DV897ECS-BV; Andor Technology, Belfast, Northern Ireland). Prism-type total internal reflection fluorescence (TIRF) microscopy was used to excite the fluorophores with a linearly polarized 532 nm laser line (I70C-SPECTRUM Argon/Krypton laser; Coherent Inc., Santa Clara, CA) focused to a $40 \times 20 \mu m$ region on fused-silica surfaces (Hoya Corporation USA, San Jose, CA). The incident angle at the fused-silica water interface was 68 to 71° with respect to the normal. The laser was pulsed with illumination intervals between 1 and 500 ms and excitation intensity between 0.3 and 2.6 kW/cm$^2$. By combining laser power and pulsing interval variations we obtained 50 to 3000 photons per PSF. A Nikon 100X TIRF objective (Nikon, 1.45 NA, oil immersion) was used in combination with a 2X expansion lens, giving a pixel size of 79 nm. At focus, the PSF image generated by a point light source with a mean emission wavelength of 580 nm and symmetric polarization has a full width at half maximum (FWHM) of $\approx \lambda/2NA = 580/2.9 \approx 200$ nm and theoretical $s_0 = \text{FWHM}/2.35 \approx 85$ nm. Including the pixelation effect [Eq. (2.7)], the measured PSF SD for our imaging system should be 88 nm. Due to random fluctuations in the emission polarization direction of streptavidin-Cy3 molecules attached to surfaces [39] and variations in focus between each measurement, we observed a range of $s_i$ values from 90 to 140
2.1 Precision analysis of standard deviation measurements

nm.

Single streptavidin-Cy3 molecules (SA1010; Invitrogen, Carlsbad, CA; 530/10 nm excitation, 580/60 nm emission) were immobilized on fused-silica surfaces by depositing 6 μL of 0.04 nM streptavidin-Cy3 powder dissolved in 0.5X TBE buffer (45 mM Tris, 45 mM Boric Acid, 1 mM EDTA, pH 7.0). The aliquot was sandwiched by a glass coverslip whose edges were sealed with nail polish. The fused-silica chips were cleaned using oxygen plasma before use. We inspected for possible surface fluorescence contaminations by imaging the TBE buffer alone; no impurities were found on either the fused-silica surface or in the buffer. The immobilization of the adsorbed molecules was verified by centroid versus time measurements.

2.1.3.2 Data acquisition and selection

Typical movies were obtained by synchronizing the onset of camera exposure with laser illumination for different intervals. The gain levels of the camera were adjusted such that none of the pixels of a PSF reached the saturation level of the camera. For the initial step, streptavidin-Cy3 monomers were first selected in IMAGEJ (NIH, Bethesda, MD) by examining the fluorescence time traces of the molecules for a single bleaching step [50]. For a selected monomer, the intensity values for 25 × 25 pixels centered at the molecule were recorded. The center 15 × 15 pixels of the PSF were used for 2D Gaussian fitting with peripheral pixels used for background analysis.

The intensity values of the selected molecules were first converted to photon counts (see below) and then fitted to the following 2D Gaussian function using a least squares
2.1 Precision analysis of standard deviation measurements

curve fitting algorithm (lsqcurvefit) provided by MATLAB (The Mathworks, Natick, MA):

\[ f(x, y) = f_0 \exp \left[ -\frac{(x - x_0)^2}{2s_x^2} - \frac{(y - y_0)^2}{2s_y^2} \right] + \langle b \rangle, \]  

(1.7)

where \( f_0 \) was the amplitude and \( \langle b \rangle \) was the mean background value. A background pixel’s total count is the sum of the floor, electronic readout noise, and background fluorescence counts. For the \( \langle b \rangle \) in this article, the floor value, determined by the lowest background pixel value, has already been subtracted. With this fitting, the PSF’s SD values in both the \( x \) and \( y \) directions, its measured location \( (x_0, y_0) \), and the image’s mean background value were obtained.

The selected streptavidin-Cy3 monomers were further characterized to satisfy the following conditions used for SD error analysis: (i) No stage drift detected by using centroid versus time measurements; stage drift introduces additional blur to each single molecule PSF and thus affects the measured SD values. (ii) A minimum of 75 valid PSF images, each with a photon count \( N \), that fluctuated less than 20% from the experimental mean \( \langle N \rangle \), of the monomer. This restriction is necessary for precise SD error analysis by using a statistically sufficient number of PSFs with consistent \( N \). (iii) PSFs with signal-to-noise ratios (SNRs, \( I_0/\sqrt{I_0 + \sigma_b^2} \)) greater than 2.5, where \( I_0 \) is the peak PSF photon count (total photon count minus \( \langle b \rangle \)) and \( \sigma_b^2 \) is the background variance in photons. (iv) Mean \( \langle s_x \rangle \) and \( \langle s_y \rangle \) obtained by Gaussian fitting of the \( s_x \) and \( s_y \) distributions of all valid images did not differ by more than 10 nm, or \( \pm 5\% \) of the mean SD value to minimize polarization effects of Cy3. (v) The mean SD values
\( \langle s_i \rangle \) were between 95 and 135 nm to minimize defocusing effects. These constraints on \( s_x \) and \( s_y \) are necessary for obtaining the expression for \( \Delta s_{\text{rms},i} \), as a function of \( N \), with minimal variations in the other parameters.

To convert from a pixel’s camera count to photons, the camera count value was divided by \( M \). In order to obtain \( M \) for each experimental setting, the center nine pixel values of the PSF were evaluated if the molecule’s average SNR was greater than 3. According to Eq. (A.4),

\[
M = \frac{(\sigma_{i}^{*2} - \sigma_{b}^{*2})}{2 (\langle N_{i}^{*} \rangle - \langle b^{*} \rangle)},
\]

(2.10)

where \( \langle N_{i}^{*} \rangle \) and \( \sigma_{i}^{*} \) are the Gaussian fitted mean and standard deviation of the measured camera count distribution of pixel \( i \), respectively. Here \( \langle N_{i}^{*} \rangle \) is the mean camera count that includes background fluorescence and electronic noise counts.

2.1.3.3 PSF and background simulations

Single molecule PSFs were generated using the Gaussian random number generator in MATLAB. For Fig. 2.2, \( s_i \) of each simulated PSF was determined by the experimental means \( \langle s_i \rangle \). The observed fluctuation in the number of photons \( N \), was incorporated. The generated photons of each PSF were binned into \( 15 \times 15 \) pixels with a pixel size of 79 nm. Each pixel’s photon count was converted into camera counts (\( M = 1 \)) using Eq. (A.1). Random background photons at each pixel were generated using the corresponding experimental background distributions. Although
2.1 Precision analysis of standard deviation measurements

the exact experimental background distributions were used for the simulations, the numerical integrations and analytical calculations were computed using the theoretical variance and the mean of all background counts, $\sigma_b^2$ and $\langle b \rangle$, respectively, rather than their fitted values. The background counts are primarily drawn from two types of distributions: a Gaussian with a high mean or a truncated Gaussian with a low mean, depending on the background fluorescence levels of each specific experiment (see Fig. 2.1 C). The final simulated PSFs with background noise were fitted to a 2D Gaussian [Eq. (1.7)] to obtain the centroid and SD values of the PSF. For each simulated $\Delta s_{x,\text{rms}}$ data point, 1000 iterations were performed and the Gaussian fitted SDs of the $s_x$ distributions were the simulated $\Delta s_{x,\text{rms}}$ results.

2.1.4 Results

We report our study for $\Delta s_{x,\text{rms}}$ using four different methods: (i) experimental measurements, (ii) simulations, (iii) numerical integrations of Eq. (2.3), and (iv) analytical calculations according to Eq. (2.9).

Figure 2.1 A shows a set of single streptavidin-Cy3 molecule images with an increasing number of detected photons $N$. These molecules have similar mean SD $\langle s_x \rangle$, values of 110, 111, and 107 nm, respectively. In order to demonstrate the decreasing SD error with increasing $N$, each representative image was chosen such that the 2D SD value was the sum of the mean SD $\langle s_x \rangle$, and one SD of the molecule’s $s_x$ distribution $\Delta s_{x,\text{rms}}$ ($\text{SD}_{\text{image}} = \langle s_x \rangle + \Delta s_{x,\text{rms}}$). To clearly illustrate the change in the SD error, which is measured as $\text{SD}_{\text{image}} - \langle s_x \rangle$, the 1D intensity profiles of the
2.1 Precision analysis of standard deviation measurements

Figure 2.1: Representative images with increasing $N$ and corresponding 1D Gaussian fits. (A) Representative images with increasing $N$ of 151, 393, and 1891 photons of single streptavidin-Cy3 molecules. (B) 1D intensity profiles (circles) of the molecules in (A) and their corresponding Gaussian fits. The respective 1D SD values are 195.4, 140.5, and 110.9 nm. The scale bar is 500 nm. (C) Background count distributions (circles) for the three molecules in (A) and their fits. The histograms are simulated background distributions which reproduce those observed experimentally.
2.1 Precision analysis of standard deviation measurements

PSFs are plotted in Fig. 2.1 as opposed to their 2D intensity profiles for clarity. The 1D intensity values were obtained by averaging transverse pixel intensity values of the PSF at each longitudinal pixel $i$. The measured 2D $SD_{image}$ values deviate from their respective means $\langle s_x \rangle$, values by 10.3, 7.2, and 2.7 nm. As expected, the 2D SD error decreases with increasing $N$.

Figure 2.2: Comparison of $\Delta s_x,rms$ versus $N$ obtained by using four different methods: experimental measurements (solid squares), simulations (circles), numerical integrations (crosses), and analytical calculations (dashed line). Each experimental $\Delta s_{x,rms}$ data point is the SD from the Gaussian fit to the $s_x$ distribution of a single streptavidin-Cy3 monomer. For each data point, its experimental $N$, $\langle s_i \rangle$, $\sigma_b$, and $\langle b \rangle$ values were used for the numerical integrations and analytical calculations. The experimental results are on average 57% higher than the analytical calculations.

Figure 2.2 shows $\Delta s_{x,rms}$ obtained by using experimental measurements, simulations, numerical integrations, and analytical calculations. Each experimental $\Delta s_{x,rms}$ data point is the SD of the $s_x$ distribution for a single streptavidin-Cy3 monomer. For
2.1 Precision analysis of standard deviation measurements

each of these data points, a simulation run for 1000 iterations was performed. The parameters were based upon experimental results including fluctuations in a PSF’s total detected photons, background distribution, and the $s_{0i}$ values determined by the mean experimental $\langle s_i \rangle$ after subtracting for the pixelation effect [Eq. (2.7)]. The finite bandwidth of the emission filter was also taken into consideration by simulating each photon as being drawn from a PSF whose width is varied according to a Gaussian distribution centered about $s_{0i}$ (with a SD of 2 nm). Numerical integrations and analytical calculations used the same $\langle N \rangle$, $s_{0i}$, $\sigma_b$, and $\langle b \rangle$ as those in the corresponding experimental data points. For all $N$, the numerically integrated $\Delta s_{x,rms}$ results are $\approx 15\%$ higher than the theoretical results while the experimental results are $\approx 57\%$ higher. The causes of these discrepancies are discussed in the following section. The simulations agree well with the experimental results.

2.1.5 Discussion

Here we discuss three issues: (i) the causes for discrepancies between results obtained using different methods, (ii) modifications to the centroid measurement error developed by Thompson, Larson, and Webb [2] to include EMCCD camera-to-photon count conversion effects, and (iii) application of our analytical expression to various single molecule studies by relating $\Delta s_{i,rms}$ to the errors of the measured quantities associated with each of the aforementioned applications.
2.1.5.1 Causes for discrepancies

Numerical integration results are consistently higher than the analytical results by 15%, while simulation (and experimental) results are higher than analytical results by 57% for all $N$. There are several reasons for these discrepancies:

a. The analytical $\Delta s_{i,rms}$ result [Eq. (2.9)] is obtained by evaluating Eq. (2.3) for the two limiting cases of $\sigma_i^2$ at the high photon count and high background noise regimes. The intermediate or transition region is absent and thus the numerical integration and simulation results tend to be larger.

b. When $N_i$ is expanded about $s_0$, the higher order terms were neglected [Eq. (A.13)].

c. In the $\Delta s_{rms}$ calculation (Appendix A.2), the $N_i$ distribution function is assumed to be a Gaussian for all pixels of the PSF [Eq. (A.5)]. This assumption will only be statistically accurate for center pixels of PSFs with high $N$. For peripheral pixels, especially for PSFs with low $N$, the $N_i$ distribution function approaches a Poisson with a low mean rather than a Gaussian. These different $N_i$ distributions, which have been verified by simulation, were not considered in either the analytical calculations or numerical integrations.

d. In simulations, we attempted to model the background count distribution exactly, whereas in numerical integrations and analytical calculations, the shape of the background count distribution was not considered, and therefore did not
influence the results.

In summary, the analytical calculation of the SD measurement error [Eq. (2.9)] is a reasonable approximation for a large range of experimental parameters. When the observed discrepancy is accounted for, the expression is in excellent agreement with our experimental results. Additional simulations suggest this difference is moderately dependent on the system’s pixel size (see Sec. 2.2.4.1) and minimized when the pixel size is comparable to the SD of the PSF as is usually the case; however, significant deviations are expected at either extrema for \( a >> s_0 \) and \( a << s_0 \).

### 2.1.5.2 Modifications to centroid error analysis

The PSF centroid error expression developed by Thompson, Larson, and Webb [2] did not take the photon-to-camera count conversion variance into consideration. Additionally, the theoretical standard deviation \( s_0 \), should be modified to include the pixelation effect \( \sqrt{s_0^2 + a^2/12} \), with respect to both directions. We have modified the PSF centroid measurement error for \( x \), to be

\[
\Delta x_{\text{rms}} = \sqrt{\frac{2(s_{0x}^2 + \frac{a^2}{12})}{N}} + \frac{8\pi(s_{0x}^2 + \frac{a^2}{12})^{3/2} (s_{0y}^2 + \frac{a^2}{12})^{1/2} (\sigma_b^2 + \langle b \rangle)}{a^2 N^2}. \tag{2.11}
\]

This analytical expression for the centroid measurement error underestimates the experimental results by 42% for the studied fluorophore and our system’s pixel size.

Figure 2.3 compares the SD measurement error \( \Delta s_{x,\text{rms}} \), to the centroid measure-
ment error $\Delta x_{rms}$, of one streptavidin-Cy3 monomer. Figure 2.3 A shows the $s_x$ and centroid location $x$, distributions of the molecule. Note that the standard deviations of the distributions, measured as $\Delta s_{x,rms}$ and $\Delta x_{rms}$, differ by 1.44 nm, with the latter being larger. Figure 2.3 B shows that the experimental $\Delta x_{rms}$ is consistently larger than $\Delta s_{x,rms}$, at various $N$, by an average of 1.25 times, indicating that the resolving power of our SD analysis is greater than that of centroid analysis for comparable experimental settings. Extrapolating $\Delta s_{x,rms}$ to high $N$ ($\approx 5000$ photons), the error reaches 1.5 nm precision.

2.1.5.3 Applications of $\Delta s_{i,rms}$ in future single molecule studies

With regards to the three applications of SD measurements previously discussed, $\Delta s_{i,rms}$ can be associated with the precisions for each of the applications’ measured quantities. Given a diffraction-limited imaging system, the maximum achievable resolution necessitates that any measured $s_i$ should be within the SD of the PSF of a visible light source $\pm \Delta s_{i,rms}$ [23, 42]. Thus, the SD measurement error directly provides the precision for quantifying an imaging system’s resolution. For molecular orientation studies, the polarized PSF can have an elliptical intensity profile that can be fitted to an asymmetric 2D Gaussian [39–41]. When an expression relating $s_x$ and $s_y$ to the orientation is developed, the errors associated with these measured quantities can be used to calculate the error associated with the reported orientation of the molecule via error propagation.

The axial ($z$) position of a single molecule is typically inferred from the measured
2.1 Precision analysis of standard deviation measurements

Figure 2.3: Comparison of $\Delta s_{x,rms}$ and $\Delta x$ versus $N$. (A) PSF SD values $s_x$ (empty bars) and centroid location $x$ (solid bars) distributions of a streptavidin-Cy3 monomer with $\langle N \rangle = 1117$ photons. The SDs of the distributions are $\Delta s_{x,rms} = 5.22$ nm and $\Delta x_{rms} = 6.66$ nm; therefore, $\Delta s_{x,rms}$ is 1.27 times smaller than $\Delta x_{rms}$. (B) Experimental $\Delta s_{x,rms}$ (solid squares) and $\Delta x_{rms}$ (circles) data along with their fits show that for the same experimental settings, the error in SD measurements is consistently lower than the error in centroid measurements. The error bars associated with the SD measurements of the $s_x$ and $x$ distributions are $\Delta s_{x,rms} \sqrt{2/N_{frames}}$ and $\Delta x_{rms} \sqrt{2/N_{frames}}$, respectively, where $N_{frames}$ is the number of fitted frames of the molecule. $\Delta s_{x,rms}$ is on average 1.25 times smaller than $\Delta x_{rms}$. The fits are theoretical results for $\Delta s_{x,rms}$ [Eq. (2.9), dashed line] shifted up by 60% and for $\Delta x_{rms}$ [Eq. (2.11), solid line] shifted up by 42%.
2.1 Precision analysis of standard deviation measurements

SD of the PSF which increases with the defocusing distance \([27, 30, 36]\). Consequently, the axial localization error \(\Delta z\), can be obtained from a single image according to error propagation, constituting a significant improvement in temporal resolution (see Sec. 2.2) \([51]\).

In addition to stationary, single molecules, SD measurements can be used to differentiate in-plane, closely separated fluorophores which may appear as dimers or larger complexes as well as address the physical properties of diffusing molecules, specifically their diffusion coefficients based on the following observations: (i) identical fluorophores separated by a distance shorter than the diffraction-limit exhibits a combined intensity profile with a SD that increases with their separation (see Sec. 2.3) \([52]\), and (ii) the intensity profile of a freely-diffusing fluorophore imaged for a finite exposure time undergoes motion-induced blurring such that the measured SD is directly proportional to the molecule’s diffusion coefficient assuming it is sampling over all space without directional bias (see Sec. 3.1) \([53]\). With modification, the method for estimating \(\Delta s_{i,\text{rms}}\) of stationary single molecules, described above, can be extended to these studies. It is possible, however, that the \(N_i\) distribution function at each pixel may be different from the Gaussian assumption for stationary molecules in Eq. (2.4). Consequently, the specific \(N_i\) distribution function for these studies can be obtained and both \(\sigma_i^2\) [Eq. (A.4)] as well as the corresponding \(\Delta s_{i,\text{rms}}\) can be derived using the same procedure outlined in the theory (Sec. 2.1.2).
2.2 Single-image axial localization analysis

2.2.1 Introduction

SPT has become a powerful tool for investigating various biological systems dependent on consecutive 3D localization measurements [35–38]. To achieve high spatial and temporal resolution, it is necessary to report the precisions of such measurements in an accurate and timely manner [3]. Two-dimensional localization of a molecule is typically accomplished by fitting its respective intensity profile to a Gaussian function thereby determining its centroid [2, 3]. However, determination of a molecule’s axial (z) position and associated error remain a challenge and demand improvements in image analysis for higher temporal resolution given a single lateral-section image. The achievable resolution along the axial dimension is dictated by the Rayleigh criterion, $2n\lambda/(NA)^2$, where $\lambda$ corresponds to the wavelength of light used, NA is the numerical aperture of the objective, and $n$ is the imaging medium’s index of refraction [27]. Attempts to circumvent this threshold have prompted the development of certain SPT techniques and localization analysis [2, 34, 44] which permit more precise measurements within the lateral plane via an analytical formula; an analogous expression for axial position measurements is currently lacking.

Among the methods capable of determining a particle’s axial position, several complicate the imaging system by either modifying the underlying PSF [54] or making

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2.2 Single-image axial localization analysis

inferences from intensity variations [35] through the use of multiple focal planes [55]. Still others utilize off-focus imaging by analyzing the appearance of diffraction rings at depths limited to greater than \( \sim 0.5 \, \mu m \) above or below the focal plane [36, 56]; tracking particles at shallower depths is possible but at the expense of increasing object size [37]. Nonetheless, the most frequently employed method determines the axial position according to the SD of a molecule’s intensity profile [27, 38, 57] and is used by a new class of ‘super-resolution’ setups [30–32, 58] such as STORM and PALM. With the exception of a few probabilistic models which can estimate a particle’s axial position within a certain confidence interval [59–61], the aforementioned techniques can only report a theoretical lower limit from Fisher matrix analysis, specifically calculating Cramér-Rao bounds [22] for the precision or localization error of their measurements. This approach, though, suffers from several disadvantages: (i) the Cramér-Rao calculation is an estimation of the lower bound relying on well-defined noise contributions which may not be known nor account for the nature of the fluorophore used and/or additional experimental parameters, (ii) any localization error derived underestimates the true value and needs to be scaled accordingly; however, this will be only approximate as there is systematic deviation due to dependences on pixelation and potentially other instrumentation effects, and (iii) when propagating the error given bounds to the SD variance, it assumes these variances are independent along each lateral dimension [2, 34, 38]. Furthermore, since an expression that includes all experimental noise parameters is unavailable, neither method can be used to assess axial-localization precisions in alternative experimental settings. While tech-
2.2 Single-image axial localization analysis

Techniques involving image stacks *i.e.*, many images collected from repeated excitations, can escape the limitations on resolution, they still require prolonged exposures to generate a sufficient number of images per molecule each containing enough photons to determine the molecules’ positions and localization errors from ensemble averaging. Use of image stacks also may not be feasible in cases where fluorophores undergo either fast diffusion and/or photobleaching. Moreover, for super-resolution studies, slow acquisition and activating fewer molecules per image compounds the time required to determine the molecules’ locations and associated precisions [38, 62].

The failure to correctly report the positional accuracy on faster timescales significantly limits the application and efficiency of numerous SPT methods. To address this problem, we have derived an analytical expression for the absolute error associated with axial localization based on SD measurements. We verify our formula from experimental studies of defocused fluorophores achieving nanometer precision. As such, single-image localization analysis can be readily incorporated into such super-resolution methods as well as standard fluorescence imaging modalities to yield comparable localization errors on the nanometer scale for both the lateral and axial dimensions; consequently, this constitutes a simplification in image analysis and a drastic improvement in temporal resolution up to two orders in magnitude to the typical exposure timescales of milliseconds.
2.2 Single-image axial localization analysis

2.2.2 Theory

A molecule’s position in two dimensions is often obtained by fitting the observed intensity profile to a Gaussian function [Eq. (1.7)] since the shape of the PSF’s central peak is irrespective of the particle’s axial position and therefore allows for consistent centroid determination. In addition to determining the centroid, its associated precision is used to validate Gaussian fitting as an accurate SPT method as well as providing a means of characterizing the achievable resolution. Although the use of image stacks would be required to assess the localization error for the centroid, ∆x, by computing the standard deviation of all measurements, an analytical approach developed by Bobroff [44] and subsequently used for centroid error analysis by Thompson, Larson, and Webb [2] allows for simple computation of the standard error of the mean given a single image. It has been previously demonstrated that nanometer precision can be achieved in the lateral dimensions from experimental verification of the reported analytical expression for ∆x and which can also incorporate additional variance due to photon-to-camera count conversion for systems using an EMCCD camera [3, 34].

While determination of a single fluorescent molecule’s centroid is easily accomplished, localizing its position along the axial dimension is not. As evidenced by Fig 2.4 A, as the depth between the molecule and the imaging plane increases, the width of the intensity profile broadens significantly and observance of diffraction rings associated with the Airy disk become more prominent thereby prohibiting accurate
2.2 Single-image axial localization analysis

determination of the object’s SD and consequently its axial position. The explanation for these diffraction patterns is attributed to the presence of spherical aberrations in the system. Prior to the appearance of diffraction rings at \( \sim 0.5 \mu m \) of the focal plane, the PSF of a defocused fluorophore is known to exhibit axial symmetry whereby the measured SD along either lateral dimension, \( s_i \), is related to the axial position or defocusing distance, \( z \), [63] in the following:

\[
s_i(z) = s_f \sqrt{1 + \left( \frac{z}{d} \right)^2 + B \left( \frac{z}{d} \right)^4},
\]

(2.12)

where \( s_f = \sqrt{s_0^2(z = 0) + \frac{a_i^2}{12}} \) is the PBS SD at focus including the pixelation effect, \( d \) is the imaging depth of the microscope, and \( B \) is a higher order fitting parameter to correct for the refractive index mismatch effect and the non-ideality of an imaging system [30, 54]. Inverting Eq. (2.12), we can solve for \( z \) as a function of \( s_i \)

\[
z(s_i) = \pm \frac{d}{B^{1/4}} \left( C - \frac{1}{2\sqrt{B}} \right)^{1/2},
\]

(2.13)

where \( C = \sqrt{\left( \frac{a_i}{s_f} \right)^2 - 1 + \frac{1}{4B}} \). By error propagation, the error in the SD measurement of an image, \( \Delta s_i \), will, accordingly, correspond to the precision with which one can determine a molecule’s position along the axial dimension, \( \Delta z = (\frac{\partial z}{\partial s_i}) \Delta s_i \). An analytical expression for \( \Delta s_i \) measured along the \( x \) axis, understood to be the rms error, is given by Ref. [34]

\[
\Delta s_x = \sqrt{\frac{s_{0x}^2 + \frac{a_i^2}{12}}{N} + \frac{16\pi \left( s_{0x}^2 + \frac{a_i^2}{12} \right)^{3/2} \left( s_{0y}^2 + \frac{a_i^2}{12} \right)^{1/2} (\sigma_b^2 + \langle b \rangle)}{3a_i^2 N^2}},
\]

(2.9)

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2.2 Single-image axial localization analysis

such that $N$ is the total number of detected photons, $a$ is the camera’s pixel size, and $\langle b \rangle$ and $\sigma_b$ are the mean and SD of the background noise, respectively. Consequently, the axial localization error, whose complete derivation with and without a higher order $B$ term can be found in Appendix B.1 [Eq. (B.1)], is calculated as

$$\Delta z = \frac{d s_i \Delta s_i}{\sqrt{2 s_f^2 C \left(2C\sqrt{B} - 1\right)^{1/2}}}.$$  \hspace{1cm} (2.14)

2.2.3 Methods

2.2.3.1 Sample preparation and imaging

In this study, we used phycobilisome (PBS, *Synechocystis* sp. PCC 6803) protein complexes for our z and $\Delta z$ investigations. PBSs are large hemi-discoidal light harvesting antenna protein complexes in cyanobacteria; the molecular weight is $\approx 10$ MDa and it measures $60 \times 30 \times 20$ nm in width, height, and length [64]. A PBS molecule contains 396 fluorophores distributed throughout the complex; consequently, it serves as an ideal emitter to meet the demands of our study due to its brightness and long fluorescence lifetime. PBSs were purified following the method described in Ref. [65]; the purified PBSs were then crosslinked according to a protocol from the Noam Adir group [66]. The PBSs were diluted in 20 mM Tris-HCl buffer (pH 8.0) to approximately 0.1 nM. Manufacturer pre-cleaned fused-silica chips were used, where isolated PBS molecules were adsorbed to surfaces at low concentration. A PBS solution of 5 $\mu$L was sandwiched between the fused-silica surface and an oxygen-plasma-cleaned
2.2 Single-image axial localization analysis

coverslip (2.2 × 2.2 cm$^2$), resulting in a 10.5 µm thick water layer. Because of the
prism-type TIRF imaging setup, our sample on the fused-silica surface is 10.5 µm
away from the coverslip surface; therefore, refractive index mismatch will affect the
PBS SD versus $z$ relation [54, 67].

Single molecule prism-type TIRF microscopy was performed as described in Sec.
2.1.3.1 using a linearly polarized 568 nm laser line with an incident angle at the fused-
silica water interface of 70° with respect to the normal. The laser excitation was pulsed
with illumination intervals of 10 ms; the excitation intensity was 5.2 kW/cm$^2$. The
excitation filter was 568/20 nm, and the emission filter was a 580 nm long pass filter.

2.2.3.2 Data acquisition and selection

PBS movies were obtained by synchronizing the onset of camera exposure with laser
illumination. The maximum gain level of the camera was used and the data acquisi-
tion rate was 1 MHz pixels/s (≈3.3 frames/s). Single molecule images were checked
such that there were no saturations in the intensity profiles. The axial position of the
objective was controlled by a focus drive (H122; Prior Scientific Inc., Rockland, MA),
moving one-way from $z \approx 700$ to $-350$ nm in 50 nm increments and 1 s intervals
while a PBS movie was recorded, taking individual snapshots at every axial location;
then, 50-image movies were acquired at the next three consecutive axial locations
(0.3 s intervals), before a last movie was recorded for $z \approx -500$ to $-750$ nm. Here,
positive defocusing distance $z$, is defined as when the surface-adsorbed PBS molecules
are on the side of the focal plane closer to the glass coverslip. For PBS analyses, 35
2.2 Single-image axial localization analysis

× 35 pixel boxes centered at the molecule were selected by hand using IMAGEJ; the center 25 × 25 pixels containing the PSF were used for 2D Gaussian fitting, and the peripheral pixels were used for background analysis. Only PBS molecules with SNRs \( \geq 2.5 \) were used for analysis.

Before analysis, the camera’s intensity count at each pixel in an image was converted into photon count by using the photon-to-camera count conversion factor calibrated the same day of the measurement as detailed in Ref. [34]. The number of detected photons in an image was obtained by subtracting the total photon count of the background from the total photon count of the image. The PBS intensity profiles were fit to a 2D Gaussian function as described in Sec. 2.1.3.2 such that \( s_x \) and \( s_y \) are the SDs in the \( x \) and \( y \) directions, respectively, \( x_0 \) and \( y_0 \) are the centroid location of the molecule, and \( \langle b \rangle \) is the mean background offset in photons.

2.2.4 Results

In Fig. 2.4 A, we show images of a single PBS molecule at different defocusing distances \( z \), separated by 350 nm; it is obvious that the size of the image (or \( s_i \)) increases as one moves away from focus. The mean \( s_x \) values from single-image measurements of six simultaneously imaged, in-plane PBS molecules for \( z \) moving from \( \approx 700 \) to \( -750 \) nm in 50 nm increments produces the symmetric \( s_x \) versus \( z \) curve in Fig. 2.4 B that is fit according to Eq. (2.12) (the \( z \) limits were determined to be before the onset of diffraction rings). While it has been previously demonstrated that nanometer precision in the lateral dimensions using localization analysis can be
achieved [2, 3, 34], the same is true for axial position measurements. From Eqs. (2.9) and (2.14), we calculated and plotted the average $\Delta s_x$ and $\Delta z$ values (including the modification to $\Delta s_x$, and consequently to $\Delta z$ by the appropriate scaling factors; see Sec. 2.2.4.1) as the $y$ and $x$ axis error bars, respectively. Since $\Delta z$ diverges at low $|z|$, only $\Delta z$ at $|z| \geq \Delta z(z)$ are shown.

In order to validate our method in obtaining $\Delta z$ by error propagation, we used image stacks by performing repeated measurements of a single PBS molecule at three axial locations near $z = -400$ nm separated by 50 nm. Figures 2.4, C and D show the $s_x$ and the corresponding $z$ values [calculated according to Eq. (2.13)], respectively, at the three axial locations. The two error bars at each axial location compare the analytical values with the experimentally determined $\Delta s_x$ and $\Delta z$ [insets to Figs. 2.4, C and D], showing agreement.

### 2.2.4.1 Theoretical $\Delta s_i$ scaling factor calculations

As discussed in Ref. [34], the analytical expression for $\Delta s_i$ [Eq. (2.9)] is known to underreport the true experimental value. As the pixel size increases, the measured SD of the PSF approaches that of the pixel’s, which is dictated by a top-hat or uniform distribution function. Eventually, at sufficiently large pixel sizes, where the whole PSF is contained within a single pixel, variations among measured SD values decrease and the associated error should be zero. Conversely, a lower limit should also exist for pixel sizes considerably smaller than the SD. As the PSF continues to spread out for increasing SDs, a pixel’s shot noise vanishes and all intensities appear
2.2 Single-image axial localization analysis
as background which begins to dominate; furthermore, the localization error is derived only to first order. Thus, the error is largely dependent on \( N \) when \( s_0 >> a \). The respective theoretical limits to the accuracy of \( \Delta s_i \) are calculated in Eqs. (2.15a) and (2.15b) determined by differentiating Eq. (2.9) with respect to \( a \) and \( s_0 \)

\[
\frac{|a|}{s_0} = \begin{cases} 
\frac{12}{9N} \frac{\sigma_b^2}{\pi} + 1 & a >> s_0, \\
\frac{12}{9N} \frac{s_0}{\pi} + 1 & s_0 >> a.
\end{cases}
\]  

\hspace{1cm} \text{(2.15a)} \hspace{1cm} \text{(2.15b)}

Since the discrepancy between the analytical theory and experiment persists and is dependent on the pixel size, specifically \( a/s_0 \), a difference among the measured SDs of the fluorophores being imaged (PBSs as opposed to Cy3 molecules) results

Figure 2.4 (preceding page): PBS axial localization and precision studies. (A) Snapshots of a single PBS molecule separated by 350 nm along \( z \) (the middle image is at \( z \approx -50 \) nm); the scale bar is 500 nm. (B) Mean \( s_x \) versus \( z \) for six simultaneously imaged, in-plane PBS molecules. The solid line is a fit to the data according to Eq. (2.12), yielding \( s_f = 144.1 \) nm, \( d = 1854 \) nm, and \( B = 220.5 \). The \( y \) and \( x \) axis error bars are the average \( \Delta s_x \) and \( \Delta z \) values of the six PBS molecules. Note that the errors increase as \( z \) decreases because the PBS molecules gradually bleached with imaging time from \( \approx 4800 \) to 1400 photons per image. (C) 50 consecutive \( s_x \) measurements for the PBS molecule in A at each of the three \( z \) locations indicated by the blue circle drawn in B (gray lines); the mean photon count per image is \( \approx 3000 \) photons. (D) The corresponding \( z \) values calculated according to Eq. (2.13) and using the above fitted parameters (gray lines). At each axial location in C and D, the black horizontal lines outline the average \( s_x \) and \( z \) values, and the left (black) and right (red) error bars represent the respective experimental and theoretical \( \Delta s_x \) and \( \Delta z \) values. Insets to C and D show Gaussian fits to the distributions of the experimental \( s_x \) and \( z \) data for the middle axial location; the SDs of the fits (experimental error bars) are \( \Delta s_x = 6.3 \) nm and \( \Delta z = 21.5 \) nm, in good agreement with the theoretical values of \( \Delta s_x = 6.0 \) nm and \( \Delta z = 20.0 \) nm.
2.2 Single-image axial localization analysis

in different scaling factors; the pixel size was fixed at 79 nm. Figure 2.5 shows $\Delta s_x$ versus $a/s_0x$ studied by simulations for $s_0x$ that ranged from 142 to 404 nm (same range as in Fig. 2.4 B). Each data point is the SD of the $s_x$ distribution of 2000 simulated PSFs using the average experimental values of $N = 3000$, $\sigma_b = 1$, and $\langle b \rangle = 4$ photons. The simulated $\Delta s_x$ results were compared to the calculated $\Delta s_x$ results, yielding a scaling factor expression of $1.35 + 0.47a/s_0x$. The upper limit to $a/s_0x$ was chosen to be 0.76, which is up to where Eq. (2.9) remains valid. Since the analytical calculation does not take this large pixelation effect into consideration, these results and those of the simulations begin to rapidly diverge beyond this point. The corresponding scaling factor for $\Delta x$, for the simulations performed in Fig. 2.5, was found to be $1.06 + 0.43a/s_0x$ with an upper limit to $a/s_0x$ of 0.71 as shown in Fig. 2.6; for a range of SDs from $s_0x = 111$ to 155 nm ($0.51 < a/s_0x < 0.71$), the fitted scaling factor expression is well approximated as simply 1.30, and at $s_0x = 118$ nm, the analytical theory underestimates the true error by 28.5%.

2.2.5 Discussion

Unlike the lateral localization error $\Delta x$, the axial localization error $\Delta z$, is dependent on position, specifically $z$. $\Delta z$ versus $|z|$ is plotted in Fig. 2.7 as a function of the number of photons $N$, for our fitted experimental parameters. Nanometer precision for $\Delta z$ can be achieved with increasing $|z|$ or $N$. While Eq. (2.12) satisfactorily describes the relationship between the measured SD and the defocusing distance for our study, as well as in live cell imaging [30], it may not be the same for other exper-
Figure 2.5: Simulation (circles) and theoretical (solid black line) $\Delta s_x$ versus $a/s_{0x}$. The dashed (red) line is the theoretical $\Delta s_x$ results multiplied by $1.35 + 0.47a/s_{0x}$ as a best fit to the simulated $\Delta s_x$ showing agreement.
Figure 2.6: Simulation (circles) and theoretical (solid black line) $\Delta x$ versus $a/s_{0x}$. The dashed (red) line is the theoretical $\Delta x$ results multiplied by $1.06 + 0.43a/s_{0x}$ as a best fit to the simulated $\Delta x$ showing agreement.
2.2 Single-image axial localization analysis

Experimental systems; furthermore, as seen in Fig. 2.7, $\Delta z$ exhibits asymptotic behavior as $z$ approaches 0 due to the axial symmetry of the PSF [Eq. (2.14)]. To address these concerns, we propose the following methods of which some have already been adopted:

a. To account for the observed asymptotic behavior, solutions such as optical astigmatism via the introduction of a weak cylindrical lens [30, 68] permits calculation of axial localization errors on the nanometer scale over all defocusing distances including those near the focal plane as evidenced in Fig. 2.8. The error is minimized and finite by measuring the axial position from the SD along the PSF’s major axis on either side of the focal plane; however, the SDs in both lateral dimensions are still required to calculate $\Delta z$ [Eq. (2.14)].

b. In lieu of Eq. (2.12), SPT and other imaging techniques may employ ad hoc terms in addition to $B$ to account for spherical aberrations and other systematic effects. Alternatively, in complicated situations, such as when the $s_x$ versus $z$ curve (Fig. 2.4 B) appears asymmetric [57], multiple functions for various regions of the curve can be used whereby $\Delta z$ is readily calculated provided $z$ can be written in terms of $s_i$ as in Eq. (2.13).

The methodology described above provides an analytical expression for the axial localization error. We have demonstrated that single-image 3D localization analysis accurately reports the precision associated with position measurements eliminating the need for image stacks. The improvement in temporal resolution consequently
Figure 2.7: PBS $\Delta z$ versus $|z|$ calculations according to Eq. (2.14) for detected photon counts $N$, of 100, 500, 1000, 5000, and $2 \times 10^4$ (top to bottom). No background noise is included. Only $\Delta z$ at $|z| \geq \Delta z(z)$ are shown.
2.2 Single-image axial localization analysis

Figure 2.8: PBS $\Delta z$ versus $|z|$ using optical astigmatism. (A) PBS $s_x(z)$ and $s_y(z)$ curves with shifted foci at $z = \pm 300$ nm using the same fitted experimental parameters as in Fig. 2.4 B. (B) Corresponding $\Delta z$ versus $z$ calculations for $N = 100, 500, 1000, 5000, \text{ and } 2 \times 10^4$ photons (top to bottom). Since $s_x$ and $s_y$ are required to obtain $\Delta z$ [Eq. (2.14)], only $\Delta z$, where both $s_x$ and $s_y$ are defined in A, are calculated. The bold lines represent the minimized $\Delta z$ values at all measurable $z$ while the other curves show only $\Delta z$ at $|z \pm 300 \text{ nm}| \geq \Delta z(z)$ for $N = 500$ and $2 \times 10^4$ photons measured along the same dimension (asymptotic and not minimized).
permits faster tracking of molecules as they can be followed reliably within the imaging depth of the microscope and therefore within the limits of Eq. (2.12); this has recently been shown from diffusion coefficient measurements of single molecules [53]. Super-resolution techniques [30–32, 58] including STORM and PALM also utilize similar expressions as Eq. (2.12) and are capable of selectively imaging multiple fluorophores, summarily obtaining 3D reconstructions to high precision from repeated excitations. Due to the tradeoff between acquisition speed and longer imaging times required for precision reporting, localization analysis can be easily incorporated with the result being an improvement in temporal resolution up to two orders in magnitude and ultimately investigations of dynamic systems in future cell imaging studies.
2.3 Single-image separation measurements of unresolved fluorophores

2.3.1 Introduction

The use of high-resolution imaging in fluorescence microscopy has permitted investigations of single molecules to macromolecular complexes on the nanometer scale. However, the ability to visualize and measure the properties of multiple fluorophores in close contact with each other under conditions of high sample concentration or formed as part of a complex, is limited by factors including the restriction of conventional far-field microscopy to measurements greater than the diffraction limit, $0.61\lambda/NA \approx 230$ nm for a mean visible wavelength $\lambda \approx 550$ nm and a microscope objective’s numerical aperture $NA \approx 1.45$ [23]. Moreover, techniques such as Förster resonance energy transfer (FRET) that take advantage of the proximity among chromophores via dipole-dipole coupling to overcome this barrier are only capable of resolving distances less than $\sim 10$ nm [15–18]. Thus, the need to observe fluorescent molecules over increasing length scales between these regimes is apparent as is the need to properly discriminate among complexes comprised of two or more molecules.

Recent single molecule fluorescence imaging methods can circumvent the Rayleigh criterion and measure subdiffraction separations with nanometer precision; however, such precision is not achieved without tradeoffs. Several techniques including

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2.3 Single-image separation measurements of unresolved fluorophores

SHRImP, NALNS, PAINT, and PALM rely on fluorophores that undergo photobleaching whereby separations can be determined from comparison of consecutive frames assuming the specimen remains static during the time series [32, 69–71]. Still others, while applicable to dynamic systems, require complicated experimental setups making use of photoswitchable or multi-color fluorescent labels such as PALM, STORM, and SHREC [32, 72–74], rendering the fluorophore and spectral selections restrictive. Additionally, as these methods are based on measuring the centroid localizations for each of the constituent molecules in the field of view, the absolute and oftentimes only relative separations that can be made are limited by the total time to satisfactorily excite all molecules or wait until all have photobleached; this typically occurs within seconds which is slower than the timescales associated with changes to molecular orientations and separations. Although SHREC allows simultaneous separation measurements between two molecules in the timescale of milliseconds, heterogenous labeling of fluorophores and overlapping images with spectrally distinct emissions pose additional challenges.

Here we report an alternative technique to analyze well-separated (\(\sim 10\) nm) molecules labeled with identical fluorophores and measure their subdiffraction separations from a single image; consequently, this represents an improvement in temporal resolution and constitutes a single-image molecular analysis (SIMA) method. For simplicity, we refer to two separated fluorophores as a dimer. The intensity profiles, for images comprising dimers and individual molecules (monomers) that were simultaneously illuminated by standard laser excitation for milliseconds exposure, were
recorded and subsequently fit to a 2D Gaussian function which reliably approximates the expected PSF for dimers separated by a distance less than the diffraction limit. While the positions of the two constituent molecules cannot be resolved, the spread of the dimer’s intensity profile, or SD, can be measured and increases with the dimer separation. The longitudinal SD (along the dimer’s major axis), the total detected photon count $N$, and other experimental parameters associated with the error in measuring the SD allow for (i) differentiating dimers from monomers, and (ii) determining the separation distance of the dimer with known precision. We have compared the experimental results with simulations performed yielding excellent agreement.

### 2.3.2 Theory

In previous super-resolution studies using highly concentrated samples, diffraction-limited spots have been observed whose widths are larger than the Rayleigh criterion due to overlapping PSFs [30, 62]. This is evidenced in Fig. 2.9 which illustrates how the measured SD of a dimer fitted by a Gaussian along its major axis varies with the separation distance $\delta$. Nevertheless, it remains a challenge to determine if one is imaging dimers as opposed to individual molecules (monomers) which are similar in appearance at short separations. The proposed method analyzes a single image such that the experimental parameters obtained from 2D Gaussian fitting can be used to differentiate dimers from monomers and subsequently report the measured separation with associated precision.

We use the criterion that if the suspected dimer’s PSF SD $s_d$, exceeds that of
2.3 Single-image separation measurements of unresolved fluorophores

the monomer’s PSF SD $s_m$, by more than the sum of the dimer and monomer SD measurement errors, $\Delta s_d$ and $\Delta s_m$, respectively, as

$$s_d - s_m > \Delta s_d + \Delta s_m,$$

then the image is likely that of a subdiffraction-separated dimer. Here, $\Delta s$ represents the SD of a distribution of measured $s_i$ values from single images. An analytical expression for the SD error of a single molecule, $\Delta s_i$, measured along the $x$ axis, is given by Ref. [34]

$$\Delta s_x = \sqrt{s_{0x}^2 + \frac{a^2}{12} + \frac{16\pi (s_{0x}^2 + \frac{a^2}{12})^{3/2} (s_{0y}^2 + \frac{a^2}{12})^{1/2} (\sigma_b^2 + \langle b \rangle)}{3a^2 N^2}}, \quad (2.9)$$

where $N$ is the total number of detected photons, $a$ is the camera’s pixel size, and $\langle b \rangle$ and $\sigma_b$ are the mean and standard deviation of the background noise, respectively. Although an exact expression for $\Delta s_d$ is lacking and can be derived in a similar fashion as in Ref. [34], Eq. (2.9) should satisfactorily account for any discrepancies associated with modeling the dimer as a Gaussian provided the appropriate SDs are used for calculations. To determine whether the differences in the SD distributions of monomers and dimers allows for adequate differentiation, a sample test intended for uneven population sizes with possibly unequal variances, i.e., Welch’s t-test, can be used.
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\[ t = \frac{\langle s_d \rangle - \langle s_m \rangle}{\sqrt{\frac{\Delta s_d^2}{2N_d \text{frames}} + \frac{\Delta s_m^2}{2N_m \text{frames}}}}, \]  

(2.17)
such that the resulting p-value sufficient to reject the null hypothesis; \( N_{\text{frames}} \) corresponds to the number of analyzed images or sample size of the \( s_i \) distribution.

We have previously demonstrated that the results for simulations of single molecule PSFs were in excellent agreement with our experimental findings [34]; consequently, PSF simulations of multiple, in-plane single molecules separated by known distances should also be accurate and verifiable by fluorescence studies. Since \( s_d \) is observed to increase with \( \delta \), a relationship between these two quantities can be established from interpolation of all measured values (in lieu of a derived equation) and the separation distance inferred from the measured SD to known precision following error propagation.

2.3.3 Methods

2.3.3.1 Sample preparation and imaging

Streptavidin-Cy3 powder was dissolved in 0.5X TBE buffer (45 mM Tris, 45 mM Boric Acid, 1 mM EDTA, pH 7.0) to make the protein solution (0.04 nM). Coverslips and fused-silica chips were cleaned using oxygen plasma. Streptavidin-Cy3 molecules were immobilized on fused-silica surfaces by sandwiching 6 \( \mu \)L of the protein solution between the surface and a coverslip whose edges were then sealed with nail polish.

Single molecule prism-type TIRF microscopy was performed as described in Sec.
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2.1.3.1 using a linearly polarized 532 nm laser line. The laser excitation was pulsed with illumination intervals between 1 and 500 ms and excitation intensities between 0.3 and 2.6 kW/cm$^2$. By combining laser intensity and pulsing interval variations we obtained 50 to 3000 detected photons per monomer PSF. The excitation filter was 530/10 nm and the emission filter was 580/60 nm.

2.3.3.2 Data acquisition and selection

Typical movies were obtained by synchronizing the onset of camera exposure with laser illumination for different intervals. The gain levels of the camera were adjusted such that none of the pixels within an image reached the saturation level of the camera. Streptavidin-Cy3 monomers were first selected in IMAGEJ by examining the fluorescence time traces of the molecules for a single bleaching step [50]. For a selected monomer, the intensity values for $25 \times 25$ pixels centered at the molecule were recorded.

Before analysis, the camera’s intensity count of each pixel was converted into photon counts by using the photon-to-camera count conversion factor calibrated previously [34]. Then, the number of detected photons, and the $x$ axis and $y$ axis dimer PSF SDs were obtained. The number of detected photons was obtained by subtracting the total photon count of the image by the total photon count of the background; the two SD values were parameters of a 2D Gaussian fit to the intensity profile of the image using Eq. (1.7) such that $s_x$ and $s_y$ are the SDs in the $x$ and $y$ directions, respectively, $x_0$ and $y_0$ are the centroid location of the molecule, and $\langle b \rangle$ is the mean
background offset in photons.

The selected streptavidin-Cy3 monomers were further characterized to satisfy the same conditions as described in Sec. 2.1.3.2 used for SD error analysis. Images from 12 monomer movies satisfied these restrictions.

2.3.3.3 Creating experimental dimer images

Experimental dimer images were constructed by adding all consecutive nondegenerate two images of a monomer movie, with one of the two images shifted 0 to 3 pixels in the $x$ direction. The final dimer images were reboxed to the center $15 \times 15$ pixels for the case of 0 nm separation (monomers) and $20 \times 20$ pixels for other separations. The intensities of the peripheral pixels from the monomer images were used for background analysis for other separations as well. By selecting the center pixels for Gaussian fitting, the non-overlapping background pixels were avoided. All experimental data in Fig. 2.10 were constructed from the 12 monomer movies with a selected separation. For the experimental data in Fig. 2.11, one monomer movie was used for all separations.

2.3.3.4 Dimer PSF simulations

Single molecule PSFs were simulated using the Gaussian random number generator in MATLAB. For simulations that were compared to experimental data (Figs. 2.10, A and B and Fig. 2.11), the simulated monomer’s SDs without the pixelation effect, $s_{m0i}$, were determined by the experimental means $\langle s_{m1i} \rangle$ [Eq. (2.7)]. The finite
2.3 Single-image separation measurements of unresolved fluorophores

bandwidth of the emission filter was also taken into consideration by simulating each photon as being drawn from a PSF whose width is varied according to a Gaussian distribution centered about $s_{m,0i}$ (with a SD of 2 nm). The experimental $N$ distribution and the restriction that only photon counts that fluctuated less than 20% from $\langle N \rangle$ were used. For simulations that were not compared to experimental data (Figs. 2.10 C and 2.12), the simulated monomer’s SDs were $s_{m,0i} = 110$ nm, the SD in photon count was 10% of the mean photon count and again only randomly generated $N$ that were within 20% of the mean were used as images. The generated photons of each PSF were binned into $20 \times 20$ pixels with a pixel size of 79 nm.

Using the simulated monomer movie, two nondegenerate consecutive images were shifted and then summed to create dimers. After construction of a dimer PSF in photons, each photon in a pixel was converted into camera counts using Eq. (2.10) with a conversion factor $M$, of one. Background noise was added to the images such that random background photons at each pixel were generated for Figs. 2.10, A and B and Fig. 2.11 using the corresponding experimental background distribution functions obtained at 0 nm separation. For Figs. 2.10 C and 2.12, the background photon distributions obtained at 0 nm separation averaged for all 12 experimental monomer movies had a mean background of 1.8 photons and a mean background SD of 1.7 photons. For each simulated dimer data point, 1000 iterations were performed such that the center $20 \times 20$ pixels of each image were used for 2D Gaussian analysis.
2.3.4 Results

As previously discussed, when two identical, in-plane fluorophores are separated by less than the diffraction limit, the SD of the dimer’s PSF increases with the separation. This is illustrated in Fig. 2.9 A which shows an array of streptavidin-Cy3 dimers with increasing separations $\delta$, of 0, 79, 158, and 237 nm in the horizontal direction. These images were constructed by combining two nondegenerate consecutive images from a single experimental monomer movie such that either of the images was initially displaced by 0 to 3 pixels (79 nm/pixel) horizontally, before being added. The number of detected photons $N$, of each monomer image was 511 photons. Whereas the first image (dimer at 0 nm separation) cannot be analyzed, at the final separation (237 nm), which is approximately equal to the diffraction limit, the image is considerably larger and the individual PSFs associated with each of the constituent molecules may be resolvable.

To demonstrate quantitatively that the SD increases with separation, the dimers’ PSFs were fit to a 1D Gaussian as presented in Fig. 2.9 B, where all transverse pixel intensity values at each longitudinal pixel were averaged. It is known that the sum of two closely separated Gaussian functions, up to a certain distance, is well described by a unimodal distribution [75]. The measured SDs increased as 114.1, 119.7, 141.6, and 178.6 nm, respectively.
2.3 Single-image separation measurements of unresolved fluorophores

Figure 2.9: Dimers of different subdiffraction separations. (A) Streptavidin-Cy3 dimers aligned in the horizontal direction separated by 0, 79, 158, and 237 nm (left to right). It is not evident from their appearance that for dimers separated by less than the diffraction limit that the images are comprised of two molecules. The scale bar is 0.5 µm. (B) 1D intensity profiles of the dimers (circles) and their corresponding Gaussian fits illustrating that the SDs increase with separation.

2.3.4.1 Differentiating dimers from monomers using a single image

Figure 2.9 illustrated that dimer images, for many separations less than the diffraction limit, are similar in appearance to those of monomers. To differentiate between the two images, we use Eq. (2.16) which provides a criterion based on the difference of the measured SDs of the dimer and monomer images, $s_d$ and $s_m$, respectively, as well as the sum of their corresponding errors, $\Delta s_d$ and $\Delta s_m$ [Eq. (2.9)]; these errors, as discussed in Ref. [34], are principally dependent on the number of detected photons $N$.

Figure 2.10 A compares the experimental and simulated SD distributions of a constructed dimer at 0 nm separation (monomer) and a dimer at 158 nm separation. The two experimental distributions were constructed from a single monomer movie,
yielding 118 valid dimer images with a photon count of $2,200 \pm 156$ (mean ± SD). The simulations used the experimental monomer $N$ and $s_m$ distributions with background photon distributions determined from those of the dimer at 0 nm separation. The simulations were run for 1000 iterations such that the counts were scaled to have the same amplitude as the experimental distributions, for comparison, in Fig. 2.10 A showing agreement.

From Gaussian fits to the experimental SD distributions in Fig. 2.10 A, the means were found to be $s_d = 139.5$ nm and $s_m = 105.2$ nm, with SDs of $\Delta s_d = 3.8$ nm and $\Delta s_m = 3.5$ nm, respectively. Likewise, results from simulations yielded means of $s_d = 138.6$ nm and $s_m = 106.7$ nm, with SDs of $\Delta s_d = 4.8$ nm and $\Delta s_m = 3.8$ nm, respectively. In accordance with Eq. (2.16), since the measured SD of the dimer,
2.3 Single-image separation measurements of unresolved fluorophores

$s_d$ exceeded $s_m + \Delta s_d + \Delta s_m = 106.7 + 3.8 + 4.8 = 115.3$ nm (using the largest obtained values), the constructed dimer at 158 nm separation for $N = 2200$ photons, is reliably shown to be a dimer with a confidence interval that can be determined from a standard t-test given by Eq. (2.17).

In general, for arbitrary separation distances, it is necessary to determine the SD measurement errors of a single image [Eq. (2.9)] which are largely dependent on the number of detected photons. Figure 2.10 $B$ shows the experimental and simulated $\Delta s_d$ results for a dimer at 158 nm separation as a function of $N$. Since the measurement error improves with the number of detected photons, so does the ability to differentiate dimers from monomers. This is illustrated in Fig. 2.10 $C$ which delineates differentiable and non-differentiable dimers at varying separations with a threshold line indicating the requisite number of detected photons. Experimental data from 12 monomer movies of different $\langle N \rangle$ (same as in Ref. [34]) each at a random separation were plotted accordingly.

2.3.4.2 Dimer separation measurement and associated error

To determine a dimer’s separation distance, it is necessary to evaluate the measured SDs of single images for known separations. This is accomplished straightforwardly by simulations of closely separated dimers; it was previously shown that simulations of single molecule PSFs yielded results in excellent agreement with experiments [34]. Figure 2.11 plots the measured dimer SD versus separation distance curve studied by simulations for various separations. The experimental results for dimers separated
by 0 to 3 pixels (79 nm/pixel) from a single monomer movie of $\langle N \rangle = 3050$ photons are also plotted. The data points represent the means of the dimer SD distributions while the corresponding error bars are their SDs. For simplicity, the solid line is a fit to the simulated data as $s_d = 0.001\delta^2 - 0.01\delta + 118$, and is independent of $N$.

![Image](image.png)

Figure 2.11: Dimer SD versus separation curve. Mean SD values from simulations of 1000 dimer images (crosses) and experimental data at separations of 0, 79, 158, and 237 nm (circles) with their corresponding error bars determined from the SDs of their respective distributions. The solid line is a fit to the simulated data as $s_d = 0.001\delta^2 - 0.01\delta + 118$. The horizontal gray lines are extensions of the SD error bars at the example data point ($\delta \approx 120$ nm) which can be used, from their intersection with the curve, to infer the associated separation measurement errors indicated by the vertical gray lines.

Each dimer separation measurement $\delta$, should have an associated precision $\Delta\delta$, which can be obtained from error propagation of the $s_d(\delta)$ fit; this is illustrated in Fig. 2.11 by the intersection of the horizontal gray lines extending from the SD error bars at the example data point with the $s_d$ versus $\delta$ curve. Consequently,
upper and lower bounds to $\Delta \delta$ are extrapolated for each separation measurement with their average being the reported error. In general, $\Delta \delta$ varies with $\delta$ as well as $N$ as shown in Fig. 2.12. Each line, representing $s_d$ versus $\delta$ simulations for different photon counts terminates on the left side at the appropriate threshold value for differentiating dimers from monomers [Eq. (2.16) and Fig. 2.10 C]. It is evident that $\Delta \delta$ decreases with $\delta$ since $s_d$ varies little at small separations according to Fig. 2.11, which therefore translates into larger separation errors; conversely, at larger separations, $s_d$ is considerably greater than $s_m$ and the corresponding separation distance is readily calculated. Eventually as the $\delta$ approaches the diffraction limit and the constituent monomers can be resolved, $\Delta \delta$ should only be dependent on $\Delta s_m$.

For $2 \times 10^4$ photons, $\Delta \delta \approx 2.3$ and 10 nm at separations of 250 and 40 nm, respectively while for 150 photons, $\Delta \delta \approx 42$ and 29 nm for the same separations.

Furthermore, we can compare these results to those obtained from centroid measurements (i.e., the error associated with measuring the separation distance from the centroids of two molecules using multi-color labeling techniques or following a photobleaching event). An upper bound for this separation error derived from centroid measurements, will be the sum of the respective centroid measurement errors [calculated using Eq. (2.11) including a 40% correction to accommodate for the discrepancy between experimental and analytical results] assuming each monomer emits half of the dimer’s total detected photon counts in addition to background noise. For $2 \times 10^4$ and 150 photons, the errors are $\approx (2 \times 1.6) \times 1.4 = 4.5$ nm and $(2 \times 30.5) \times 1.4 = 85.3$ nm, respectively, and illustrated by the lower and upper dashed lines in Fig.
2.3 Single-image separation measurements of unresolved fluorophores

2.12 (the values also agree with another estimation in Ref. [76]).

![Graph showing dimer separation measurement error versus separation for detected photon counts \(N\), of 150, 300, 400, 600, 2000, \(2 \times 10^4\), and \(2 \times 10^4\) (top to bottom). Each of the curves terminates on the left side at the appropriate threshold value for differentiating dimers from monomers. The horizontal dashed lines are the upper bounds to the dimer separation measurement error derived from using the centroid method for \(N = 2 \times 10^4\) (bottom) and 150 (top) photons.]

Figure 2.12: Dimer separation measurement error versus separation for detected photon counts \(N\), of 150, 300, 400, 600, 2000, \(2 \times 10^4\), and \(2 \times 10^4\) (top to bottom). Each of the curves terminates on the left side at the appropriate threshold value for differentiating dimers from monomers. The horizontal dashed lines are the upper bounds to the dimer separation measurement error derived from using the centroid method for \(N = 2 \times 10^4\) (bottom) and 150 (top) photons.

2.3.5 Discussion

The procedure presented above offers an approach for discriminating between closely separated molecules and single fluorophores. This method can be implemented in most experiments and is advantageous over existing techniques since only a single type of fluorophore or dye is required thereby avoiding the drawbacks common to multi-color labeling including registration problems among different images. Furthermore, the benefit of no longer waiting for a molecule to undergo photobleaching can
2.3 Single-image separation measurements of unresolved fluorophores

dress many of the challenges working with specific fluorophores and their expected fluorescence lifetimes; dimer separations can be computed from a single image on the timescale of milliseconds representing an improvement in temporal resolution. Although there is typically a tradeoff between temporal and spatial resolution, the precisions associated with dimer separation measurements using SIMA to those obtained from centroid measurements as in Fig. 2.12, are comparable.

All the aforementioned techniques assume that the two molecules are primarily localized to the imaging plane; molecules located within the imaging depth of the microscope (≈200 nm) appear in focus with minimal change to their measured SDs and thus measurements of separation distances well below the diffraction limit are possible. Nevertheless, several limitations persist such as the inability to measure separation distances, of candidate dimers, less than approximately the width of a pixel unless the number of detected photons is high. For the case of a highly efficient emitter and/or longer exposure time yielding \( N = 2 \times 10^4 \) photons, the separation distance has a lower limit of \( \approx 40 \) nm. Typical experimental photon counts from 100 to 2000 photons per image restricts the range of measurable dimer separations to be between the pixel size and the diffraction limit but should meet the demand of many biological and nano-scientific applications. Potential extensions include differentiating complexes comprised of more than two molecules and measuring the relative separations between them.
Chapter 3

Analyzing proteins’ diffusive properties with high temporal resolution

Certainly no subject is making more progress on so many fronts than biology, and if we were to name the most powerful assumption of all, which leads one on and on in an attempt to understand life, it is that all things are made of atoms, and that everything that living things do can be understood in terms of the jiggling and wiggling of atoms. -Richard Feynman, Lectures on Physics (1963)

Diffusion coefficient measurements are important for many biological studies including reaction kinetics and particle size determinations. Among current methods, single particle tracking is capable of measuring the diffusion coefficient while preserving a
molecule's spatial information between imaging times using only femtomoles of sample. However, the associated temporal resolution is limited to seconds and limits the method's ability to address fast diffusive processes, e.g., proteins' three-dimensional diffusion in solution. By directly imaging fluorescent proteins and studying the widths of their intensity profiles, we can determine their respective diffusion coefficients to known precision by analyzing single images obtained using TIRF microscopy at sub-millisecond exposure times. The method is easily adapted to other imaging modalities and applications in basic research and pharmaceutical investigations such as fast drug screening are envisioned. Our theoretical formulation agrees with results from experiments and simulations for a protein of known diffusion coefficient.

3.1 Single-image diffusion coefficient measurements

3.1.1 Introduction

Determination of particles’ diffusion coefficients is important for many biological and material applications, such as single molecule dynamics studies [4, 77, 78], biochemical and pharmaceutical reaction kinetics [79, 80], and particle size and shape determinations [81]. Among current methods for measuring diffusion coefficients, such as NMR [82], dynamic light scattering [83], fluorescence correlation spectroscopy (FCS) [11–13], and fluorescence recovery after photobleaching (FRAP) [14], single particle

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tracking (SPT) offers the unique capability for determining locations and diffusion coefficients simultaneously. This is essential for investigating various molecular processes in heterogeneous environments such as inside a cell’s cytoplasm [84], flagella [85], membrane [86], or protein-nucleic acid interactions in vitro [4]. Because of this capability, and the additional advantage that SPT experiments require less than femtomoles of sample, SPT can be a powerful tool for measuring diffusion coefficients in a large number of biological investigations in vivo and in vitro in which supplies are scarce.

Nevertheless, SPT is known to suffer from low temporal resolution thereby limiting the range of measurable diffusion coefficients. In single molecule fluorescence imaging studies, the intensity profiles of stationary or slowly moving (relative to the data acquisition timescales), regarded as point spread functions (PSFs), are fit to 2D Gaussian functions to determine the molecules’ localization information. The centroid from the Gaussian fit directly measures the probable position of the molecule in the lateral dimensions at the time of imaging, whereas the standard deviation (SD) is used to infer its axial location. In SPT diffusion coefficient measurements, consecutive measurements of a fluorophore’s locations are used to calculate the diffusion coefficient following mean square displacement analysis [4, 84, 87]. At least 20 data points are required from each single trajectory to adequately estimate the respective diffusion coefficient; with current single-photon camera imaging rates of \( \sim 100 \) frames/s for a finite-sized imaging area, the minimum data acquisition time is 0.2 s and three-dimensional (3D) diffusion coefficient \( (D_{3D}) \) measurements up to order \( 10^5 \)
nm²/s have been reported [88]. However, this time is insufficient to measure diffusion coefficients of highly mobile molecules, such as nanometer-sized proteins that diffuse beyond the typical imaging depth of ∼400 nm of single molecule studies in <1 ms (a typical 5 nm protein with $D_{3D} \approx 10^8$ nm²/s, diffuses $\sqrt{2D_{3D}t} \approx 447$ nm in 1 ms).

Recently, measurements of $D_{3D}$ up to $1.7 \times 10^7$ nm²/s have been reported using a SPT method that relies on particles labeled with two colors [89]; however, multi-color labeling may not be feasible for many biological systems of interest, which restricts the applicability of the method.

Consequently, the ability to measure 3D diffusion coefficients of single-colored, nanometer-sized particles in their native environment is highly desirable for in vivo and in vitro SPT studies. In order to capture the molecule within the microscope’s imaging depth, the imaging time needs to be less than 1 ms. Here we report a novel (to our knowledge) method that can be used to determine diffusion coefficients of highly mobile Brownian molecules from the measured SDs of their intensity profiles in sub-millisecond exposure times. As an extension from our previous study of stationary fluorophores [34] to dynamic ones, this method relies on single-image molecular analysis (SIMA) in order to probe the physical properties of molecules, specifically their diffusion coefficients. In this study we used enhanced green fluorescent protein (eGFP) for measurements and analyses.

Because the imaging times of our method are <1 ms, the temporal resolution for diffusion coefficient measurements is improved by at least 1000-fold over FCS (multiple measurements, each ∼20 s long), 200-fold over conventional SPT, 50-fold over
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FRAP, and 10-fold over two-color SPT. Furthermore, the improvement in temporal resolution, by requiring only a single image for analysis, is achieved without compromising the precision to $D_{3D}$ measurements, while avoiding the drawbacks associated with fluorescence imaging including photobleaching and limited lifetime photons. Below, we describe our method which relates the SD of a freely diffusing protein’s intensity profile to its 3D diffusion coefficient $D_{3D}$. In a previous study, Schuster et al. [90] used a similar concept to relate small 2D diffusion coefficients (up to $1.1 \times 10^6$ nm$^2$/s with a temporal resolution $> 25$ ms) to a fluorophore’s spot size. Here, we extend that study to fast 3D diffusions ($D_{3D}$ up to $>10^8$ nm$^2$/s and temporal resolutions $< 1$ ms). Accordingly, and by providing the explicit conditions for measuring particles of different sizes (i.e., the appropriate exposure time for a particular particle size; see Appendix C.6), our study allows for $D_{3D}$ determination of highly mobile molecules in their native environments.

3.1.2 Theory

The ability to extract a molecule’s 3D diffusion coefficient $D_{3D}$, from a single image is premised on the fact that a diffusing fluorophore exhibits an intensity profile that is broader compared to that of a stationary molecule as illustrated in Fig. 3.1. Molecules with larger diffusion coefficients are then expected to have intensity profiles with increasing SDs due to motion-induced blurring for a given exposure time $t$. However, as $t$ decreases, a particle will, on average, sample less space thereby reducing the intensity profile’s measured SD along with its associated precision since fewer photons
are detected. Consequently, an equation for the SD as a function of $D_{3D}$ and $t$ can be established.

Since a molecule has a characteristic point spread function $f(x, y, z)$, specific to the experimental system used, its corresponding intensity profile, projected onto a 2D imaging plane, represents a convolution of the PSF with its pathway distribution function (PWDF) $g(x, y, z, t)$,

$$I(x, y) \propto f(x, y; z) \ast g(x, y; z; t). \quad (3.1)$$

For a stationary point light source imaged through a circular aperture, the PSF is an Airy disk but it is traditionally modeled by a 2D Gaussian function because it is more mathematically tractable with negligible expected differences in practice. The SD in either the $x$ or $y$ directions, $s_i$, of the PSF for a single fluorescent molecule imaged at a distance $z$ from the focal plane was previously shown to obey Eq. (2.12) which is simplified to first order as

$$s_i(z) = s_f \sqrt{1 + \left( \frac{z}{d} \right)^2}, \quad (3.2)$$

where $s_f = \sqrt{s_0^2(z = 0) + a^2}$ is the PSF SD at focus including the pixelation effect and $d$ is the imaging depth of the microscope ($\sim 400$ nm). Furthermore, total internal reflection fluorescence (TIRF) microscopy introduces an evanescent wave described by a decaying exponential, $z_d^{-1}e^{-z/z_d}$, where $z_d$ is the penetration depth ($\sim 100$ nm), designed to excite molecules closer to the glass-water interface thereby influencing
3.1 Single-image diffusion coefficient measurements

the PSF and subsequently the intensity profile particularly for the case of a freely diffusing molecule that emits photons along its entire trajectory within the exposure time.

A pathway distribution function is used to characterize the times a molecule spends at any given location. Since a freely diffusing particle undergoes Brownian motion, there is no model that accurately reflects the PWDF for the duration the molecule is being excited. However, the probability distribution function (PDF) for a particle’s likely position at some later time $t$, is known to be Gaussian with a variance equal to the mean square displacement and is easily verified. Considering a particle’s trajectory over all times, the PDF describing the spread of its locations can be treated as another Gaussian with a variance $\propto D_{3D}t$; while the PDFs describing single particles’ trajectories are inherently different and may not be viewed as Gaussians, the cumulative PDF of multiple particle trajectories can be. PWDFs in the axial and lateral directions (see Appendix C.2) are calculated with proportionality factors $A_i$, determined from simulations of freely diffusing particles; particles were released along the axial dimension according to the PDF of starting positions at different exposure times to account for the expected photon distributions and a boundary condition at $z = 0$ (i.e., the glass-water interface) which they are subject to (see Appendix C.1).

While any single particle trajectory will have a corresponding intensity profile (and associated SD) that is unique, the measured SD should, nonetheless, belong to its limiting distribution and approach the distribution’s mean at longer exposure times. Consequently, the intensity profile for the expected trajectory of a single
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The particle is a convolution of the approximations, above, for the PSF and PWDFs,

\[
I = \frac{1}{2\pi s_x s_y \sqrt{\pi D_3 t}} \exp \left\{ -\left( \frac{x^2}{2s_x^2} + \frac{y^2}{2s_y^2} \right) \left[ 1 - \frac{1}{1 + \left( \frac{d}{z} \right)^2} \right] - \frac{z}{z_d} \right\} \exp \left\{ -\left[ \frac{x^2 + y^2}{2A_x D_3 t} + \frac{(z - \langle z_0 \rangle)^2}{2A_z D_3 t} \right] \right\}, \tag{3.3}
\]

in which we have explicitly used the pathway distribution function in the \(x\) direction \(PWDF_x\), for both lateral dimensions (since it is isotropic) with a mean of zero, and permitted elliptical PSFs. The convolution in the \(x\) and \(y\) dimensions yields

\[
I = \frac{1}{\pi s_x s_y \sqrt{2A_x D_3 t}} \exp \left\{ -\frac{1}{2} \left[ \frac{x^2}{s_x^2} + A_x D_3 t (1-a) \right] \left[ \frac{y^2}{s_y^2} + A_y D_3 t (1-a) \right] \right\} \exp \left\{ -\left[ \frac{(z - \langle z_0 \rangle)^2}{2A_z D_3 t} + \frac{z}{z_d} \right] \right\}, \tag{3.4}
\]

where \(a = \left[ 1 + \left( \frac{d}{z} \right)^2 \right]^{-1}\). The SD in either the \(x\) or \(y\) dimensions can be defined as

\[
\sigma_i = \sqrt{\frac{s_i^2 + A_i D_3 t (1-a)}{(1-a)}}, \tag{3.5}
\]

such that the final intensity profile will be an integration of Eq. (3.4) with respect to \(z\) from zero to infinity,
3.1 Single-image diffusion coefficient measurements

\[ I(x, y) = I_0 \int_0^\infty \frac{1}{\sigma_x \sigma_y} \exp \left\{ - \left[ \frac{x^2 \sigma_y^2 + y^2 \sigma_x^2}{2 \sigma_x^2 \sigma_y^2} \right] \right\} \exp \left\{ - \left[ \frac{(z - \langle z_0 \rangle)^2}{2 A_z D_{3D} t} + \frac{z}{z_d} \right] \right\} dz, \]

where \( I_0 = \left( \frac{\pi^{3/2} s_x s_y \sqrt{A_x A_z D_{3D} t z_d}}{2} \right)^{-1} \). Since no analytical solution exists, an approximate one can be obtained by numerically integrating Eq. (3.6) for the appropriate limits. The result is a 2D intensity profile that is, once again, well described by a Gaussian function. The corresponding equation relating the SD to \( D_{3D} \) and \( t \) does not directly follow from Eq. (3.5) but can be established from interpolation of many numerical integrations for varying \( D_{3D} t \) as discussed in the results (Sec. 3.1.4). The diffusion coefficient will therefore be a function of the measured SD and the exposure time. The possibility of background noise has been excluded from this theoretical treatment, however, it can be incorporated by convolving Eq. (3.6) with an additional Gaussian function with a variance of \( \sigma_b^2 \) and adding a constant offset for the floor value.

3.1.3 Methods

3.1.3.1 Sample preparation and imaging

eGFP molecules (4999-100; BioVision, Mountain View, CA) were diluted in 0.5X TBE buffer (45 mM Tris, 45 mM Boric Acid, 1 mM EDTA, pH 8.0) to 0.03 nM. For stationary eGFP studies, manufacturer-pre-cleaned fused-silica chips (6W675-575 20C; Hoya Corporation USA, San Jose, CA) were used, and isolated eGFP molecules
were adsorbed to surfaces at low concentration. For diffusing eGFP studies, the manufacturer-pre-cleaned fused-silica chips were treated using oxygen plasma for 3 min, rendering them hydrophilic to prevent eGFP adsorption [91]. The hydrophilic fused-silica surface can be considered ballistic for the diffusing eGFP molecules in our experiments and simulations. For both studies, a protein solution of 5 µL was sandwiched between the fused-silica surface and an oxygen-plasma-cleaned coverslip (2.2 × 2.2 cm$^2$), resulting in a 10.5 µm thick water layer. Because the oxygen-plasma-treated fused-silica surface is hydrophilic, the buffer quickly wetted the surface and bubbles were rarely observed. The coverslip edges were then sealed with nail polish to prevent possible stray flow of the buffer due to evaporation.

Single molecule imaging was performed using a Nikon Eclipse TE2000-S inverted microscope (Nikon, Melville, NY) in combination with a Nikon 100X objective (Nikon, 1.49 NA, oil immersion). The samples were excited by prism-type TIRF microscopy with a linearly polarized 488 nm laser line (I70C-SPECTRUM Argon/Krypton laser; Coherent Inc., Santa Clara, CA) focused on a 40 × 20 µm$^2$ region. The 488 nm line was filtered from the multiline laser emission with the use of polychromatic acousto-optic filters (48062 PCAOM model; NEOS Technologies, Melbourne, FL). The laser excitation was pulsed with an illumination interval of 30 ms for the stationary eGFP molecules shown in Fig. 3.1, and between 0.3 and 1 ms for the diffusing eGFP molecules. The excitation intensities were 2.7 and 3.2 kW/cm$^2$ for the respective stationary eGFP molecules, and 37.5 kW/cm$^2$ for the diffusing molecules. Images were captured by an iXon back-illuminated electron multiplying charge coupled device.
3.1 Single-image diffusion coefficient measurements

(EMCCD) camera (DV897ECS-BV; Andor Technology, Belfast, Northern Ireland). An additional 2X expansion lens was placed before the EMCCD, producing a pixel size of 79 nm. The excitation filter was 488/10 nm, and the emission filter was 525/50 nm.

3.1.3.2 Data acquisition and selection

Movies were obtained by synchronizing the onset of camera exposure with laser illumination for different intervals. The maximum gain level of the camera was used and the data acquisition rate was 1 MHz pixels/s (≈3.3 frames/s). Single molecule images were checked to ensure that there were no saturations in the intensity profiles. For the defocusing analysis of stationary eGFP molecules, 21 × 21 pixel boxes centered at the molecule were selected by hand using IMAGEJ (NIH, Bethesda, MD), and the intensity values were used for 2D Gaussian fitting. For the diffusing eGFP molecule movies, all visible diffusing eGFP intensity profiles in the peak laser excitation region of 10 × 10 µm² were selected by hand using 39 × 39 pixel boxes centered at the molecule. The center 25 × 25 pixels of the boxes were used for 2D Gaussian fitting, and the peripheral pixels were used for experimental background analysis.

Before performing the analysis, the camera’s intensity count at each pixel in an image was converted into photon count by using the photon-to-camera conversion factor calibrated the same day of the measurement as described in Ref. [34]. The number of detected photons in an image was calculated by subtracting the total photon count of the background from the total photon count of the image.
eGFP intensity profiles were fit to a 2D Gaussian function to obtain the SD values of the molecule:

\[
f(x, y) = f_0 \exp \left[ -\frac{(x - x_0)^2}{2s_x^2} - \frac{(y - y_0)^2}{2s_y^2} \right] + \langle b \rangle,
\]

where \(f_0\) is the amplitude, \(s_x\) and \(s_y\) are the SDs in the \(x\) and \(y\) directions, respectively, \(x_0\) and \(y_0\) are the centroid location of the molecule, and \(\langle b \rangle\) is the mean background offset in photons.

For the defocusing eGFP analysis, we selected 17 adsorbed eGFP molecules with a minimum photon count of 229 and signal-to-noise ratios (SNRs, \(I_0/\sqrt{I_0 + \sigma_b^2}\) > 3.75, where \(I_0\) is the peak PSF photon count (after subtracting the mean background offset \(\langle b \rangle\)) and \(\sigma_b^2\) is the background variance in photons. For the diffusing eGFP molecules, we used a SNR of 2.5 as a selection criterion, and PSFs with photon counts less than 50 were not used in the analysis. At each exposure time, we acquired 1600 data points from four movies (two were acquired at different regions of an imaging chip on the same day, and two were acquired from different chips on other days). The number of images of diffusing eGFPs used for the experimental analysis that satisfied the SNR criteria varied from 419 to 1066 for the 0.3 to 1 ms exposure times, respectively.

### 3.1.3.3 Diffusing eGFP PSF simulations

We simulated 3D Brownian diffusion eGFP trajectories at a range of exposure times using FCS-determined eGFP \(D_{3D} = 8.86 \times 10^7\) nm\(^2\)/s and triplet-state statistics.
3.1 Single-image diffusion coefficient measurements

The starting locations of the trajectories followed the distribution function described in Appendix C.1. The step sizes in the $x$, $y$, and $z$ directions were randomly selected from a Gaussian distribution with a mean of zero and SD of $\sqrt{2D_{3D}t_0}$ with a step time $t_0 = 1 \mu s$. Because of the reflective fused-silica-water interface, the simulated $z$-values were maintained above zero. The number of steps in a simulation was $t/t_0$.

At each $x$, $y$ location in a trajectory, when the molecule was not in a triplet dark state, a Poisson distributed number of photons (Appendix C.4) were drawn from a Gaussian PSF spatial distribution with a mean of zero and a corresponding SD value based on the axial location (Appendix C.3).

The simulated photons of each trajectory were binned into a $50 \times 50$ pixel region with a pixel size of 79 nm. The photon count of each pixel was converted into the modified camera count using Eq. (4) in Ref. [34] with the photon multiplication factor of the camera set to $M = 1$ to include photon-to-camera count conversion variance. Random background photons at each pixel were generated using the corresponding experimental background distribution functions for the exposure time [34]. The final intensity profiles were fit to a 2D Gaussian function to obtain the two SD values for the image. For each data point in Fig. 3.4, 1000 independent trajectories were simulated.

3.1.4 Results

As previously discussed, the method is based on the observation that the intensity profile of a diffusing molecule appears broader than that of a stationary or immobile
molecule for a finite exposure time. Figure 3.1 A shows a 30 ms frame image of immobile eGFP molecules adsorbed on a fused-silica surface while Fig. 3.1 D is of diffusing eGFP molecules near a hydrophilic fused-silica surface [91]. The corresponding intensity profiles are displayed in Fig. 3.1, B and E, respectively, such that Gaussian fits to each of the selected intensity profiles yields SDs which are consistently larger for those of diffusing molecules.

Figure 3.1: Comparison of stationary and diffusing eGFP molecules. (A) An image of stationary eGFP molecules adsorbed on a fused-silica surface. Five of the seven molecules have a SNR > 2.5. (B) Intensity profiles of the stationary molecules in panel A in photon counts. (C) Intensity profile (dots) and corresponding Gaussian fit (mesh) to the stationary eGFP molecule denoted by the arrows in A and B with a SNR of 9.8, $s_x = 107.2$ nm, and $s_y = 107.9$ nm. (D) Diffusing eGFP molecules near a reflective hydrophilic fused-silica surface at 1 ms exposure time. Six of the eight molecules have a SNR > 2.5. The scale bars for A and D are 2 µm. (E) Intensity profiles of the diffusing eGFP molecules in D. (F) Intensity profile (dots) and corresponding Gaussian fit (mesh) of the diffusing eGFP molecule denoted by the arrows in D and E with a SNR of 3.5, $s_x = 202.2$ nm, and $s_y = 192.4$ nm. It is evident that the intensity profiles of diffusing molecules are broader (or have larger SDs) than those of stationary molecules.
3.1 Single-image diffusion coefficient measurements

In general, the intensity profiles of diffusing molecules as in Fig. 3.1 represent the sum of emitted photons projected onto a 2D imaging screen from single diffusion trajectories. Figure 3.2 A shows a simulated eGFP diffusion trajectory for an exposure time of 0.6 ms (using a time step of 0.005 ms for clarity). The data are grayscaled to correspond to the particle’s axial locations (Appendix C.4). The emitted photons \(N = 414\) photons, after photon-to-camera count conversion, were projected down and binned according to the camera’s pixel area of \(79 \times 79\) nm\(^2\) (Fig. 3.2 B, bottom, gray image) with the intensity profile formed in the colored plot above. Figure 3.2 C shows the corresponding 2D Gaussian fit to the intensity profile, yielding SD values in the \(x\) and \(y\) directions.

Figure 3.2: Simulated image formation and analysis of a diffusing eGFP molecule. (A) Trajectory of a diffusing eGFP molecule in free solution under TIRF evanescent excitation at an exposure time of 0.6 ms. The data are grayscaled to correspond to the particle’s axial locations (Appendix C.4). (B) The emitted photons along the trajectory form an intensity profile (top, colored plot), which is projected onto a 2D imaging screen (bottom, gray image). (C) Intensity profile (dots) and corresponding Gaussian fit (mesh) of the diffusing eGFP molecule with \(s_x = 119.4\) nm, and \(s_y = 142.2\) nm.

To determine \(D_{3D}\) from single images of diffusing fluorophores, we performed experimental measurements, analytical calculations, and simulations for exposure times of 0.3 to 1 ms. Figure 3.3 A shows representative eGFP images (chosen such that
3.1 Single-image diffusion coefficient measurements

Each of the molecule’s $s_x$ values were within $\pm 5 \text{ nm}$ of the means of their respective SD distributions in Fig. 3.3 B) acquired from experiments at exposure times of 0.3, 0.7, and 1 ms. As expected, the $s_x$ values increase with exposure time and whose results are validated from comparison to theoretical calculations and simulations, below, yielding excellent agreement.

![Figure 3.3: Single images of diffusing eGFP molecules and their corresponding SD distributions at different exposure times. (A) Three representative images of diffusing eGFP molecules at exposure times of 0.3, 0.7, and 1 ms with $s_x$ values of 136.4, 160.9, and 175.5 nm, respectively. The scale bar is 1 $\mu$m. (B) SD distributions of eGFP intensity profiles (normalized by counts for comparison) at the three aforementioned exposure times and their corresponding Gaussian fits yielding increasing values of 136.8 $\pm$ 27.7 (mean $\pm$ SD), 159.0 $\pm$ 32.2, and 172.1 $\pm$ 34.8 nm, respectively.](image)

In analytical calculations, we deduce an expression relating the measured SD of a diffusing eGFP’s intensity profile to its 3D diffusion coefficient, $D_{3D}$. The intensity profile represents a convolution of the imaging system’s PSF with the molecule’s pathway distribution function PWDF, with the latter describing the distribution of particle locations within a single trajectory. Due to the presence of the glass-water...
3.1 Single-image diffusion coefficient measurements

interface, we must decompose eGFP’s 3D diffusive motion into two components for calculation of the PWDF and ultimately the intensity profile: 1D diffusion along the axial dimension and 2D diffusion within the lateral dimensions as in Eq. (3.1).

Furthermore, it is known that as the defocusing distance between the fluorophore and the focal plane increases, so does the PSF’s SD. Consequently, calculation of the intensity profile necessitates integrating over all axial positions the molecule may have traveled during the exposure time to obtain an axial-direction-projected PSF, \( f(x, y) \). Because diffusion in the lateral and axial dimensions are statistically independent of each other, we choose to perform this integration prior to convolving the resulting PSF \( f(x, y) \), with the PWDF associated with the molecule’s locations within the lateral dimensions, \( g(x, y) \).

\[
I(x, y) = \left( \int_{\text{PSF}(x, y, z) \cdot \text{PWDF}_z dz}^{f(x, y)} \right) \ast \text{PWDF}_{x,y}. \quad (3.7)
\]

We summarily compute the axial-direction-projected PSF by numerically integrating over all defocused PSFs with respect to \( z \),

\[
f(x, y) = \int_0^{400} C(z) \exp \left[ -\frac{x^2}{2s_x(z)^2} - \frac{y^2}{2s_y(z)^2} - \frac{(z - \langle z_0 \rangle)^2}{2A_z \cdot 2D_3D t} - \frac{z}{zd} \right] dz, \quad (3.8)
\]

where \( D_{3D} = 8.86 \times 10^7 \text{ nm}^2/s \) is determined from FCS (Appendix C.5), while \( C(z) \) and \( s_i(z) \) are the amplitude and SDs of defocused eGFP PSFs [Appendix C.3 and
3.1 Single-image diffusion coefficient measurements

Eq. (3.2)], respectively. \( C(z) \) prohibits an integration from zero to infinity as discussed in the theory (Sec. 3.1.2), consequently, Eq. (3.8) is only integrated up to the microscope’s imaging depth of 400 nm; the PWDF\(_z\) is also slightly modified according to Appendix C.2.1. While the defocused PSF is complicated by the presence of additional nonlinear terms due to the decaying TIRF evanescent intensity, the resulting axial-direction-projected PSF \( f(x, y) \), is well approximated by a Gaussian function with a SD \( s'_f \), that should be dependent on several experimental parameters, \( s'_f(s_f, d, D_{3D}, t, A_z, d) \). From analysis of eGFP \( f(x, y) \)s at different exposure times, a functional form for \( s'_f \) dependent on \( D_{3D} \) and \( t \) is assumed such that

\[
s'_f(D_{3D}t) = \sqrt{111^2 + 0.0634D_{3D}t} \approx \sqrt{s^2_f + 0.0634D_{3D}t} \text{ nm},
\]

where 111 nm and 0.0634 are fitted values.

Given that \( f(x, y) \) [at focus and Eq. (3.8)] and \( g(x, y) \)s (determined from simulations in Appendix C.2.2) are both Gaussian functions, their convolution in the lateral directions [Eq. (3.7)], is described by another Gaussian with a variance equal to their respective variances added in quadrature

\[
s_i = \sqrt{s^2_f + A_x \cdot 2D_{3D}t}.
\]  

(3.9)

Appendix C.2.2 demonstrates that for eGFP PSFs at focus with \( s_f = 108.2 \) nm convolved with PWDF\(_x\)s for an exposure time of 0.6 ms, the SD along either lateral dimension of the resulting intensity profiles should be

\[
s_i = \sqrt{s^2_f + A_x \cdot 2D_{3D}t} = \sqrt{108.2^2 + 96.8^2} = 145.2 \text{ nm},
\]

which is close to the cumulative SD distribution’s
3.1 Single-image diffusion coefficient measurements

mean of 147.1 nm.

Rearranging Eq. (3.9), the diffusion coefficient from a single image of a molecule’s intensity profile can be determined as

\[ D_{3D} = \frac{s_i^2 - s_f^2}{(2A_x + 0.0634)t}, \] (3.10)

such that if the molecule is constrained to diffuse solely within the lateral dimensions of the xy plane, the equation is truncated whereby 0.0634t vanishes \((A_z = 0)\).

For simulations of intensity profiles of diffusing eGFP molecules (as shown in Fig. 3.2), the FCS-determined \(D_{3D}\) was used. Figure 3.3 A juxtaposes the mean \(s_x\) results (and corresponding errors) from simulations and experimental measurements (as shown in Fig. 3.3 B) yielding excellent agreement at all exposure times (Fig. C.6 compares the results at an exposure time of 0.6 ms). The analytical results according to Eq. (3.9) using the FCS-determined \(D_{3D}\) are also plotted in Fig. 3.4 A; however, for longer exposure times \((t > 0.8 \text{ ms})\) these results begin to diverge since eGFP molecules diffuse beyond the diffraction limit of this study (see Appendix C.6).

The precision to the measured \(D_{3D}\) from single images of diffusing eGFP molecules, can be obtained from error propagation of Eq. (3.10)

\[ \Delta D_{3D} = \frac{s_i}{(A_x + 0.032)t} \Delta s_i, \] (3.11)

where \(\Delta s_i\) is the error associated in measuring the SD of the intensity profile \([i.e., \text{the experimental error bars in Fig. 3.4 A or Eq. (2.9)}]\) [34]. Figure 3.4 B compares
3.1 Single-image diffusion coefficient measurements

Figure 3.4: Comparison of $s_x$ and $D_{3D}$ results. (A) Mean $s_x$ versus $t$ from experimental measurements (circles), simulations (disks), and theoretical calculations (squares) for intensity profiles of diffusing eGFP molecules. For the experimental and simulated results, the error bars are the SDs of the $s_x$ distributions. (B) $D_{3D}$ versus $t$ from experimental measurements [calculated according Eq. (3.10)]. The error bars are $\Delta D_{3D}$ values [calculated according to Eq. (3.11)]; the dashed line is eGFP’s FCS-determined $D_{3D}$ of $8.86 \times 10^7$ nm$^2$/s for comparison.
3.1 Single-image diffusion coefficient measurements

$D_{3D}$ (with corresponding error bars as $\Delta D_{3D}$) from experimental measurements to eGFP’s FCS-determined $D_{3D}$, showing agreement.

At an exposure time of 0.7 ms, $\Delta D_{3D} = 5.2 \times 10^7$ nm$^2$/s for a single eGFP image [Eq. (3.11)] with $\langle s_i \rangle = 162.1$ nm and $\Delta s_i = 39.2$ nm, which is 57% of eGFP’s FCS-determined $D_{3D}$. Although this seems large, it is comparable to the error associated with FCS $D_{3D}$ measurements [13], which use highly concentrated samples, when you account for the observation that a typical frame from a SPT movie contains 30 molecules (eGFP images) on average thereby improving the method’s precision by a factor of $\sim \sqrt{30}$ to 10%. Additionally, the use of efficient emitters or higher excitation intensities would yield more detected photons $N$, during the exposure time, minimizing both $\Delta s_i$ and $\Delta D_{3D}$. In spatially restrictive environments, such as in vivo imaging of micron-sized cells, where only one image is obtained at a time, repeated measurements will enable a precise determination of $D_{3D}$.

3.1.5 Discussion

Although this study addressed fast, freely diffusing molecules several nanometers in size with $D_{3D} > 5 \times 10^7$ nm$^2$/s, the method described can be applied to most diffusive processes regardless of the native environment. For large particles that diffuse slowly due to macromolecular crowding, such as in cells [92], or from viscous solvents, the intensity profiles will be more localized and longer exposure times should be used to observe noticeable changes to the SDs. The appropriate exposure times are determined according to the study’s diffraction limit (see Appendix C.6). Under
certain cases, particle diffusion may deviate from unbiased Brownian motion (e.g., diffusion with drift or directional motion); however, anisotropic diffusion coefficients are calculable from the respective SDs measured along each of the intensity profile’s dimensions, independently or for more complicated systems, the specific PWDFs can be determined prior to solving Eq. (3.8).

In summary, we provide a new (to our knowledge) method to determine the $D_{3D}$ of fast, freely diffusing particles with high precision. The technique is premised on the observation that the intensity profile of a diffusing molecule appears broader than that of an immobile molecule; consequently, the measured SD is used to infer a molecule’s diffusion coefficient from a single image greatly improving the temporal resolution over existing methods without sacrificing visualization and tracking. It is easily adapted to other imaging modalities and applications in basic research and pharmaceutical investigations, such as fast drug screening, are envisioned.
Chapter 4

Applications of SIMA for studying biological systems

This [double helix] structure has novel features which are of considerable biological interest... It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material. -James D. Watson and Francis Crick, “Molecular Structure of Nucleic Acids”, in Nature (1953)

Single molecule studies and analysis of many biological systems including: (i) protein-DNA interactions, (ii) energy transfer within the phycobilisome, and (iii) intraflagellar transport and entry mechanisms in *Chlamydomonas*, is possible using image deconvolution techniques we have developed. One of the principal concerns of the lab and in the field of protein-nucleic acid interactions is to understand how DNA-binding
proteins locate their target sites on DNA. Several mechanisms have been proposed, according to the facilitated diffusion model, such that proteins are believed to undergo one-dimensional sliding and three-dimensional random walks or hops. Currently, single molecule studies lack the temporal resolution required to distinguish either of these diffusive processes; consequently, we performed Monte Carlo simulations in order to calculate accurate sliding diffusion coefficients from previous single molecule experiments. Moreover, the simulations establish the proper time scales future studies need to assess hopping (and sliding) kinetics.

Initial observations from single molecule investigations of the energy transfer mechanism in phycobilisomes confirms that light is emitted variedly throughout the complex contrary to the belief that it occurs solely at the terminal pigments with high quantum efficiency. This is supported by additional evidence indicating the presence of discrete ‘quenching subunits’, each comprising a group of fluorophores acting as a single quantum system, which can be localized with SIMA methods. Finally, in vivo studies of membrane signaling proteins in Chlamydomonas, many of which are conserved in humans and linked to a number of ciliopathies, are important for understanding intraflagellar transport that is associated with motility and signal transduction; specifically, we address the flagellar entry mechanism. From mean square displacement analysis of individual Pkd2-GFP trajectories, we elucidate the diffusive process, predicted according to two competing models, as well as determine the proteins’ effective diffusion coefficients. Suggestive, albeit preliminary, results reveal anomalous diffusion within the entry region consistent with one of the models.
4.1 Protein sliding and hopping kinetics on DNA

4.1.1 Introduction

Timely target association of DNA-binding (DB) proteins is important for prompt cellular response to external stimuli using mechanisms such as gene regulation, DNA replication, and DNA repair. The target association rates of DB proteins frequently deviate from the diffusion limit due to their interactions with nonspecific DNA via facilitated diffusion [4, 94, 95]. Facilitated diffusion primarily consists of two processes: sliding, where a protein diffuses along nonspecific DNA (nsDNA) without losing contact, and hopping, where the protein dissociates from DNA and undergoes three-dimensional (3D) diffusion before rebinding to the same (Fig. 4.1) or a different segment of DNA (referred to as intersegmental transfer). Here, we regard events comprising long hopping distances, usually called jumping, as a form of hopping. A DB protein may slide and hop many times on nsDNA before reaching its target site.

In order to quantify the effect of facilitated diffusion on DB proteins’ target binding rate, how long a protein spends sliding on DNA (mean sliding time $\langle t_1 \rangle$) and how fast it moves along DNA (sliding diffusion coefficient $D_1$) are two critical parameters for all calculations of in vivo and in vitro DNA configurations [95–100].

Single molecule fluorescence imaging studies of DB proteins’ Brownian diffusion along elongated DNA have obtained effective diffusion coefficients $D_{\text{eff}}$, for whole

\footnote{Material for this section comes from a published manuscript, Ref. [93]: DeSantis, M. C., J.-L. Li, and Y. M. Wang, 2011. Protein sliding and hopping kinetics on DNA. Phys. Rev. E 83:021907.}
Figure 4.1: Schematic for a diffusion trajectory showing a protein initially binding to DNA, proceeding to slide (light disks) and hop (dark disks), and finally permanently dissociating from DNA. This example diffusion trajectory has two discernible hops.

seconds-long diffusions (each observed diffusion event between protein association and permanent dissociation is defined as a diffusion trajectory with a total time, \( t \)) [4, 84, 101–112]. In the past, numerous studies had substituted \( t \) and \( D_{\text{eff}} \) values in the place of \( \langle t_1 \rangle \) and \( D_1 \), respectively, for target binding rate and protein-nsDNA binding energy calculations since the latter parameters could not be experimentally determined [4, 84, 97–100, 103, 108, 113]. Recent evidence suggests that these diffusion trajectories include both sliding and hopping: (i) the sliding time of DB proteins has been estimated to be milliseconds [98, 103, 113, 114]; (ii) the sliding displacement has been estimated to be less than 50 bp [115], shorter than the displacements of whole diffusion trajectories for the reported DB proteins (>100 nm); and (iii) hops longer than 200 nm have been observed [106]. Since the extent of hopping is unknown, it is dubious to use \( t \) and \( D_{\text{eff}} \) values in lieu of \( \langle t_1 \rangle \) and \( D_1 \), respectively. In order to obtain \( \langle t_1 \rangle \) and \( D_1 \) from experimental data, deconvolving sliding and hopping from
individual diffusion trajectories is necessary.

### 4.1.2 Theory and simulations

Here, we deconvolve sliding and hopping in a diffusion trajectory and obtain $\langle t_1 \rangle$ and $D_1$ using (i) Monte Carlo simulations, (ii) experimental $D_{\text{eff}}$ and $t$ values, and (iii) the following two relations$^2$:

\begin{align}
  t &= N \langle t_1 \rangle + N \langle t_3 \rangle , \quad (4.1a) \\
  2D_{\text{eff}} t &= 2D_1 N \langle t_1 \rangle + 2D_3 N \langle t_3 \rangle , \quad (4.1b)
\end{align}

where $N$ is the mean number of sliding and hopping alternations in a diffusion trajectory, $D_3$ is the 3D diffusion coefficient of the protein, and $\langle t_3 \rangle$ is the mean hopping time. From hopping simulations, we first determine $N$ and $\langle t_3 \rangle$, which subsequently permits calculation of $t_1$ and $D_1$ using experimental $D_{\text{eff}}$ and $t$ values in Eqs. (4.1a) and (4.1b).

For each hopping simulation, a protein was initially positioned at the protein-center-to-DNA-center distance of $R = r_{\text{DNA}} + r_{\text{protein}} + \Delta r$, where $r_{\text{DNA}} = 1$ nm is the radius of DNA, $r_{\text{GFP-LacI}} = 2.68$ nm is the hydrodynamic radius of the protein, and $\Delta r \approx 0.5$ nm is an estimate of the protein-DNA binding distance (or location of

$^2$The DB protein’s displacement on DNA, $x$, comprises displacements from alternations between 1D sliding, $x_1$, and 3D hopping, $x_3$. $x = \sum_{i=1}^{N} x_{1i} + \sum_{j=1}^{N} x_{3j}, \langle x^2 \rangle = \sum_{i=1}^{N} \langle x_{1i}^2 \rangle + \sum_{j=1}^{N} \langle x_{3j}^2 \rangle + \sum_{i,j=1}^{N} 2\langle x_{1i}x_{3j} \rangle = N \langle x_1^2 \rangle + N \langle x_3^2 \rangle = 2D_1 N \langle t_1 \rangle + 2D_3 N \langle t_3 \rangle$. This relation has also been verified by simulations.
the Debye-Hückle interaction potential minimum corresponding to an effective Debye length beyond which we consider no protein-DNA interactions) [116, 117]. The protein immediately dissociated from DNA and underwent 3D diffusion until rebinding to DNA, at which time the position was recorded, or until the maximum number of steps of the hopping simulation was reached in which case the protein was assumed to have permanently dissociated and its diffusion trajectory was not used for data analysis. Figure 4.2 describes the criterion for determining whether a hopping protein collided with DNA. For every step, the length of the perpendicular drawn from the center of the DNA to the line connecting the last two protein locations (dashed arrow) was calculated and if less than $R$, association occurred (see Appendix D.1). The binding position was chosen to be the midpoint between the two protein locations. We have modeled DNA as an infinite, rigid cylinder assuming 100% probability for association on protein-DNA collision; the distance between the protein binding location and its origin denotes the hopping distance.

Figure 4.2: Schematic for determination of protein-DNA association. The gray (open) circle marks the effective protein-DNA binding distance. The protein moves ballistically between consecutive steps.

The simulations’ parameters were determined as follows. The hopping simula-
tion step size $\delta$, and step time $\tau$, are the collision distance and time in a Brownian Dynamics scheme, respectively [118], whereby $\delta$ is chosen such that the resulting diffusion coefficient approaches its accepted value within the diffusive limit. At temperature $T = 294$K, the instantaneous velocity of a protein of mass $m$, in solution is the root mean square (rms) velocity $\sqrt{\langle v^2 \rangle} = \sqrt{k_B T/m} = \delta/\tau = 6.02$ m/s, where $k_B$ is the Boltzmann constant, and $m = 67.5$ kDa for a GFP-LacI monomer. Using the Einstein-Stokes relation, $D_3 = \delta^2/(2\tau) = k_B T/6 \pi \eta r = 8.03 \times 10^7$ nm$^2$/s for GFP-LacI where the viscosity of water is $\eta = 10^{-3}$ N s/m$^2$ and the protein’s hydrodynamic radius $r = 2.68$ nm assuming a typical protein density of 1.38 g/cm$^3$, we obtain $\delta = 2D_3/\sqrt{\langle v^2 \rangle} = 0.267$ Å and $\tau = 4.46$ ps. A simulation step along each dimension was drawn from a Gaussian distribution with a mean of zero and a standard deviation of $\delta$.

The time limit of each simulated GFP-LacI hop was $\approx 1$ ms (or $2.1 \times 10^8$ steps), selected according to the following two estimations: (i) Since the observed diffusion of proteins on DNA is the combination of sliding and hopping with diffusion coefficients $D_1$ and $D_3$, respectively, the maximum total hopping time of a diffusion trajectory should not exceed $N t_{3,\text{max}} = D_{\text{eff}} t/D_3$ when $D_1 \approx 0$ [Eq. (4.1a)]. For GFP-LacI, $\langle D_{\text{eff}} \rangle \approx 2 \times 10^4$ nm$^2$/s [4] which dictates that $t_{3,\text{max}} \approx 0.25$ ms when $t$ is on the order of 1 s and using the lower bound to $N$ of one hop per diffusion trajectory. Therefore, a hopping time limit of $t_{3,\text{max}} \approx 1$ ms for a single hop should be sufficiently long for all diffusing proteins to return to DNA. (ii) A longer time limit, such as 10 ms per hop (data not shown), results in additional proteins returning to DNA with
individual hopping distances longer than $\sqrt{2\langle D_{\text{eff}} \rangle t} = 200$ nm, a detectable distance in single molecule measurements that are usually used to separate individual diffusion trajectories into segments free of large displacements for accurate $D_{\text{eff}}$ analysis [4, 106].

4.1.3 Results

For $4 \times 10^5$ GFP-LacI hopping simulations (maximum simulation time of $t_{3,\text{max}} \approx 1$ ms) with $\delta = 0.267$ Å and $R = 4.2$ nm, 99.809% of these trials resulted in the protein reassociating to DNA and thus the probability for a simulated hop to return to DNA is $P = 0.99809$. The hopping characteristics are shown in Figs. 4.3, A and B in which the mean hopping distance along DNA is 3.37 Å (median, 0.41 Å), the mean hopping height (the maximum radial distance of the protein from DNA) is 4.93 Å (median, 0.45 Å), and the mean number of steps per hop is $4.97 \times 10^4$ (median, 5), yielding a mean hopping time of $\langle t_3 \rangle = 0.22$ µs. The mean number of hops in a GFP-LacI diffusion trajectory is $N = 526$, obtained by dividing the total number of simulated hops of $4 \times 10^5$ by the total number of unreturned hopping simulations of 763; the distribution for the number of hops per diffusion trajectory is shown in Fig. 4.4. This set of values has been verified to converge with that from a larger simulation size consisting of $4 \times 10^6$ hops; specifically, $N$ values differ by 0.57%. The inset to Fig. 4.3 B shows the distribution of total hopping displacements in a diffusion trajectory with each data point simulated from 526 randomly selected hopping displacements. The rms total hopping displacement per diffusion trajectory is $127.5$ nm ($\sqrt{2D_3N\langle t_3 \rangle}$), and the mean total hopping time is $N\langle t_3 \rangle = 115$ µs. Note that although shorter hopping
4.1 Protein sliding and hopping kinetics on DNA

distances, such as ones less than DNA’s base pair length of 3.4 Å, do not carry direct biological significance nor do they noticeably disrupt sliding, they are important for correctly assessing rms total hopping displacement statistics in a diffusion trajectory.

![Figure 4.3](image)

Figure 4.3: Hopping characteristics for $R = 4.2$ and 10.2 nm. (A) Distributions of hopping distances along DNA for $\delta = 0.267$ Å and $R = 4.2$ (green, open circles) and 10.2 nm (red dots), with hopping height for $R = 4.2$ nm (gray line). (B) Distributions of number of steps per hop for $R = 4.2$ and 10.2 nm. Inset shows the distribution of total hopping displacement per diffusion trajectory and corresponding Gaussian fit. (C) Number of hops per diffusion trajectory longer than 0.25 Å, and up to hops longer than 800 nm, for $R = 4.2$ and 10.2 nm. The crosses are experimental data for EcoRV proteins, where the occurrence rate of hops per diffusion trajectory longer than 200 nm are 0.06, 0.1, and 0.16 (the 0.15 value was omitted for clarity) [106]. (D) GFP-LacI total diffusion time ($t$) distribution (from experimental data in Ref. [4]). The mean of the exponential fit is 10.4 s.

We can also compute the ‘diffusion to capture’ probability $P$, for a protein to return to DNA using a steady-state solution to the diffusion equation, incorporating a cutoff radial distance $c$ [118]. Proteins released after the initial step at $b = 4.22$
4.1 Protein sliding and hopping kinetics on DNA

Figure 4.4: Distribution of number of hops per diffusion trajectory. The results of $4 \times 10^5$ individual hopping simulations constitute a total of 763 protein diffusion trajectories such that 526 hops occur on average per trajectory.

nm are either adsorbed at the DNA surface ($R = 4.2$ nm) or escape beyond $c = R + \sqrt{4D_3 t_{3,\text{max}}}$. The probability is time-independent and given by

$$P = \frac{\ln(c/b)}{\ln(c/R)} = 0.99896.$$ \hspace{1cm} (4.2)

Imposing the same cutoff distance $c = 551.2$ nm in subsequent simulations, we obtained $P = 0.99865$, in near agreement with the analytical value above. The derivation of Eq. (4.2) and initial release distance $b$ (averaged over all possible steps from DNA), are provided in Appendix D.2.

Having obtained $\langle t_3 \rangle$ and $N$ from simulation, we now solve Eqs. (4.1a) and (4.1b) for $\langle t_1 \rangle$ and $D_1$ given the experimentally measured values of $t$ and $D_{\text{eff}}$. With values of $D_{\text{eff}}$ for GFP-LacI ranging from $2.3 \times 10^2$ to $1.3 \times 10^5$ nm$^2$/s [4] and $t = 10.4$ s
4.1 Protein sliding and hopping kinetics on DNA

(Fig. 4.3 D),

$$\langle t_1 \rangle = \frac{t}{N} - \langle t_3 \rangle \approx \frac{t}{N} = 19.8 \text{ (ms)}, \quad (4.3a)$$

$$D_1 \approx D_{\text{eff}} - \frac{D_3 N \langle t_3 \rangle}{t} = D_{\text{eff}} - 895.1 \text{ (nm}^2/\text{s}). \quad (4.3b)$$

The sliding time is several tens of ms and $D_1$ ranges from $\approx 0$ for slow diffusion to $\approx D_{\text{eff}}$ for fast diffusion. The $\langle D_1 \rangle$ for GFP-LacI is $9.1 \times 10^3 \text{ nm}^2/\text{s}$ using $\langle D_{\text{eff}} \rangle$ of $2 \times 10^4 \text{ nm}^2/\text{s}$. Since $D_1 > 0$, Eq. (4.3b) sets the lower bound to $D_{\text{eff}}$ such that it must be greater than $D_3 N \langle t_3 \rangle / t = 895.1 \text{ nm}^2/\text{s}$. The rms total sliding displacement in a diffusion trajectory becomes longer than the rms total hopping displacement when $D_{\text{eff}} > 2 N D_3 t_3 / t \approx 1790 \text{ nm}^2/\text{s}$.

Since our protein-nsDNA binding distance is an estimate, we have carried out simulations with $\Delta r$ ranging from 0.5 to 6.5 nm (corresponding to protein-DNA distances $R$, of 4.2 to 10.2 nm, respectively). Comparing the results for $R = 10.2$ nm to $R = 4.2$ nm, the distributions of hopping distances (Fig. 4.3 A) and hopping times (Fig. 4.3 B) are similar, although the mean hopping distance reduces to 2.82 Å, the mean number of steps per hop reduces to $3.23 \times 10^4$, and the mean number of hops $N$, doubles to 1101. Solving for $\langle t_1 \rangle$ and $D_1$ for $R = 10.2$ nm, we find $\langle t_3 \rangle = 0.14 \mu\text{s}$, $N \langle t_3 \rangle = 154 \mu\text{s}$, $\langle t_1 \rangle = 9.4 \text{ ms}$ (approximately half of the value for $R = 4.2$ nm), and $D_1$ to be comparable to the previously calculated value for $R = 4.2$ nm. Given that the sliding and hopping kinetics for $R$ of 4.2 and 10.2 nm are similar, our method and results can be safely applied to most DB protein-DNA binding distances.
4.1 Protein sliding and hopping kinetics on DNA

To investigate hopping distances within a diffusion trajectory, Fig. 4.3 C shows the distribution of the number of hops per diffusion trajectory longer than a finite hopping distance, ranging from 0.25 Å to 800 nm, for \( R \) of 4.2 and 10.2 nm. For \( R = 4.2 \) nm, 3.37 hops in a diffusion trajectory were longer than 5 nm, and 11\% of diffusion trajectories had a hop longer than 200 nm. As expected, the results for \( R = 10.2 \) nm are approximately twice as large since \( N \) is doubled. The crosses represent EcoRV proteins, which have a comparable hydrodynamic radius \( r \), of 2.66 nm (see Table 4.1), that were experimentally observed in different buffers to have hopped longer than 200 nm with reported occurrences ranging from 6 to 16\% per diffusion trajectory [106]. These observations are in agreement with our simulations; furthermore, for hops longer than 300 and 500 nm, our results agree with those reported in Fig. 4 A of Ref. [106].

<table>
<thead>
<tr>
<th>Protein</th>
<th>( r_{\text{protein}} ) (nm)</th>
<th>( \delta ) (Å)</th>
<th>( D_{\text{eff}} ) (( \text{nm}^2/\text{s} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP-LacI, 2(^a)</td>
<td>3.13</td>
<td>0.284</td>
<td>( 4.6 \times 10^4 ) [103]</td>
</tr>
<tr>
<td>GFP-LacI</td>
<td>2.68</td>
<td>0.267</td>
<td>( 2.3 \times 10^2 - 1.3 \times 10^5 ) [4]</td>
</tr>
<tr>
<td>EcoRV, 2</td>
<td>2.66</td>
<td>0.262</td>
<td>( 0.9 - 2.5 \times 10^4 ) [106]</td>
</tr>
<tr>
<td>EcoRV(^b)</td>
<td></td>
<td></td>
<td>( 3.1 \times 10^3 ) [110]</td>
</tr>
<tr>
<td>RNAP, 4(^b)</td>
<td></td>
<td></td>
<td>( 6.1 \times 10^3 - 4.3 \times 10^5 ) [104]</td>
</tr>
<tr>
<td>RNAP(^b)</td>
<td></td>
<td></td>
<td>( 1.3 \times 10^5 ) [119], ( \sim 10^4 ) [101]</td>
</tr>
<tr>
<td>hOgg1</td>
<td>2.36</td>
<td>0.247</td>
<td>( 5.78 \times 10^5 ) [84]</td>
</tr>
<tr>
<td>p53</td>
<td>2.34</td>
<td>0.246</td>
<td>( 3.01 \times 10^5 ) [108]</td>
</tr>
<tr>
<td>UL42</td>
<td>2.63</td>
<td>0.261</td>
<td>( 5.1 \times 10^3 - 2.2 \times 10^4 ) [107]</td>
</tr>
<tr>
<td>T7 gp5, 2</td>
<td>2.86</td>
<td>0.272</td>
<td>( 8.0 \times 10^5 - 1.86 \times 10^6 ) [112]</td>
</tr>
<tr>
<td>T7 gp5, 2</td>
<td>3.00</td>
<td>0.278</td>
<td>( 4.0 \times 10^5 ) [112]</td>
</tr>
<tr>
<td>C-Ada</td>
<td>1.77</td>
<td>0.214</td>
<td>( 1.3 \times 10^6 ) [111]</td>
</tr>
</tbody>
</table>

\(^a\) The number 2 indicates a dimer, and 4 indicates a tetramer.

\(^b\) Unknown molecular size due to unspecified/uncertain protein components and/or labels.
4.1 Protein sliding and hopping kinetics on DNA

Other DB proteins may differ from GFP-LacI in their sizes, and thus $\delta$ and $R$. Table 4.1 lists multiple DB proteins known to diffuse by hopping (instead of proteins that slide exclusively [102]) studied using single particle tracking (SPT) methods on elongated DNA. Despite differences in $R$ up to 1.26 nm, the $\delta$ values differ only by less than 0.07 Å. These effects are considered in Fig. 4.5 A, in which the number of hops per diffusion trajectory longer than a finite distance, ranging from 0.1 Å to 800 nm for $\delta = 0.267$ Å and $R$ from 4.2 to 10.2 nm, are shown. The number of hops per diffusion trajectory increases with $R$ moderately for all hopping distances, indicating that the hopping kinetics are applicable to most observed DB proteins.

![Figure 4.5: Distributions of number of hops per diffusion trajectory longer than 0.1, 0.34, 1, 5, 10, 20, 50, 100, 200, 300, 500, and 800 nm (top to bottom in A), (A) for $R$ ranging from 4.2 to 10.2 nm (left to right) and (B) for $R = 4.2$ nm and $\delta = 0.267$ (circles), 3.4 (empty squares), and 10.2 Å (crosses). Inset shows distributions of hopping distances for the three $\delta$ values.]

The step size $\delta$, in the current approach, based on microscopic Brownian random walk models, can be made larger or smaller for vastly different particle sizes. Figure
4.1 Protein sliding and hopping kinetics on DNA

4.5 B shows distributions of hopping distances for three $\delta$ values of 0.267, 3.4, and 10 Å for $R = 4.2$ nm and $t_{3,\text{max}} \approx 1$ ms. The distribution curves collapse when the protein’s hopping distances are larger than $\delta$, indicating that the tail distribution of the probability for hopping has the same asymptotic form at long distances, in agreement with the solution to the diffusion equation [120]. However, the mean hopping distance (inset to Fig. 4.5 B; values 3.37, 36, and 95 Å), the mean number of hops $N$, in a trajectory (526, 42, and 14), and $\langle t_3 \rangle$ (0.22, 3.1, and 9.2 $\mu$s) all depend on $\delta$ sensitively, as short-length scale motions dominate protein-DNA reassociation (Fig. 4.3 A). This regime cannot be accessed in the macroscopic theory, i.e., by solving the diffusion equation directly.

4.1.4 Discussion

When the protein-nsDNA association probability $p$, is not 100%, e.g., due to rotation of the DNA-binding domain during large hops, hopping statistics and the subsequent sliding statistics will change. For a low binding probability of $p = 10\%$, although on average, ten consecutive hops would be needed for reassociation, the mean number of association attempts will still be $N$. However, the effective mean hopping time $\langle t_3' \rangle$, and the mean hopping distance are expected to increase while the effective number of hops per diffusion trajectory $N'$, decreases since $t$ is held constant. The effective total hopping time $N'(t_3')$, and the rms total hopping distance per diffusion trajectory should therefore remain constant as well. The binding probability is thus inversely related to the effective mean sliding time $\langle t_1' \rangle$, according to Eq. (4.1b) which for
4.1 Protein sliding and hopping kinetics on DNA

$p = 10\%$ results in a 10-fold increase in $\langle t'_1 \rangle$.

When the salt concentration is varied, $p$ and $R$ change, as might $D_3$ within a few angstroms from DNA. However, since $t$ remains $\approx N'(t'_3)$ because $N'(t'_3) \ll N'(t'_1)$ and that the rms total hopping displacement in a diffusion trajectory is influenced by $R$ only moderately, the observed changes in $t$ and $D_{eff}$ are likely due to sliding, rather than hopping. Consequently, these observations as a result of varying the salt concentration are not indicative of hopping and should not be used to determine its presence in diffusion trajectories, in disagreement with Refs. [84, 98, 107, 108, 112].

Some studies use flow to elongate DNA and/or investigate hopping properties of DB proteins [84, 108, 111, 112, 121]. Here, we describe the effect of flow on hopping distances using the maximum reported flow rate in SPT studies of 100 $\mu$m/s. For our mean hopping time of $\langle t_3 \rangle = 0.22 \mu$s, a typical dissociated protein is carried by flow a length 0.22 Å along DNA; this distance is negligible compared to its mean hopping distance of 3.37 Å (the total displacement of the protein from flow alone within a diffusion trajectory consisting of 526 hops will be 11.6 nm which is substantially less than the total hopping displacement of 127.5 nm observed for GFP-LacI and similarly other proteins, as shown above). On the other hand, for a trajectory that includes a hop 1 $\mu$m in length, which occurs once every 1000 diffusion trajectories, the hopping time is 6.22 ms and flow carries the protein 622 nm along DNA; this distance would be sufficient for the protein to be considered dissociated. According to Fig. 4.3 C, the probability for such an event to occur is approximately one percent of all diffusion trajectories; consequently, a protein is unlikely to have been ‘washed out’ suggesting
that flow may be inadequate for analyzing diffusion trajectories of proteins that hop, thereby contradicting the assumptions of Refs. [111, 112].

Furthermore, sliding kinetics are not expected to be drastically affected by DNA configuration since a protein remains in contact with nsDNA and should not be subject to DNA condensation and coiling either in vivo or in vitro as well as long-range forces, contrary to hopping kinetics. The reported values for $D_1$ and $t$ can therefore be applied under in vivo situations for better estimation of target binding rates.

In summary, this study analyzes DB proteins’ hopping on elongated DNA to address sliding kinetics. Using Monte Carlo simulations, we deconvolved the sliding and hopping kinetics of GFP-LacI proteins from their experimentally observed seconds-long diffusion trajectories suggesting the following: (i) in each diffusion trajectory, a protein makes on average hundreds of alternating slides and hops with a mean sliding time of several tens of ms, (ii) sliding dominates the rms displacement of fast diffusion trajectories, whereas hopping dominates slow ones, (iii) flow and variations in salt concentration have limited effects on hopping kinetics, while DNA configuration is not expected to influence sliding kinetics; and (iv) the rate of occurrence for hops longer than 200 nm agrees with experimental data for EcoRV proteins. While we have made several assumptions regarding the nature of protein association and modeling DNA, the observed sliding kinetics appears to be a robust feature. Although hopping kinetics will change according to in vivo conditions, the lower bound to $D_{\text{eff}}$ for a typical DB protein should help future experiments in identifying the presence
of hopping in diffusion trajectories with greater certainty.
4.2 Single molecule investigations of energy transfer mechanisms within the phycobilisome

4.2.1 Introduction

Cyanobacteria is a phylum of bacteria that uses photosynthesis to convert solar energy into biomass-stored chemical energy. It is ubiquitous in nature, being found in almost all conceivable environments, and accounts for roughly 20 to 30% of Earth’s total photosynthetic productivity. Visible light between 500 and 650 nm is collected by phycobilisomes (PBSs), the light harvesting complexes that reside on the surface of the thylakoid membrane. As shown in Fig. 4.6, PBSs consist of core cylinders made of stacks of allophycocyanin (APC) disks with rods radiating outward from the core comprised of phycocyanin (PC) and, when present, phycoerythrocyanin (PEC) and phycoerythrin (PE) at the periphery. In Synechocystis sp. PCC 6803, a model organism for our proposed single molecule studies, the PBS is hemi-discoidal with three...
4.2 Single molecule investigations of energy transfer mechanisms within the phycobilisome

core cylinders, each containing four trimeric APC disks (APC trimers) and six rods, each composed of three hexameric PC disks (PC hexamers). The center cavities of the PC disks are suggested to be connected by linker polypeptides [123, 124]. PC and APC are both phycobiliproteins (PBPs) that contain phycocyanobilin, one of several types of phycobilins (PBs), a chromophore especially efficient for absorbing blue light and which is also responsible for the distinct color associated with cyanobacteria; the peak absorption wavelengths for PC and APC are 620 and 650 nm, respectively.

Energy initially absorbed by PC in the rods is funneled to two terminal pigments within the APC core through what is believed to be the radiationless Förster mechanism and ultimately transferred, at a longer wavelength, to the reaction centers of photosystems II and I (PSII and PSI) for further energy conversion for cellular intake; cyanobacterial chlorophyll (Chl) within these reaction centers gathers light outside of the PBS’s absorption spectrum which is inaccessible by it. Figure 4.7 A illustrates the PBS and its association with PSs on and embedded within the thylakoid membrane.

Results from fast laser kinetics measurements and Förster calculations, based on the crystal structures of PBPs [126], suggest the energy transfer efficiency from any light absorbing PBPs to the core was 95% for the PBSs of *Synechocystis* 6701 [127]. Förster resonance energy transfer (FRET) is a mechanism describing energy transfer mediated by nonradiative dipole-dipole coupling among neighboring donor and acceptor chromophores typically separated less than 10 nm and should therefore occur between the following PBS molecular units: (i) the α84 and β155 PBs within each monomer of a PC disk [128], (ii) the PBs within each monomer of an APC
4.2 Single molecule investigations of energy transfer mechanisms within the phycobilisome

Figure 4.7: PBS and its association with PSs in the thylakoid membrane. (A) Photosynthetic system of *Synechocystis* 6803 comprising PSII dimers, PSI trimers [125], and a single PBS anchored to the thylakoid membrane. (B) EM-averaged image of the PBS showing an end view of the core cylinders and a side view of the flexible rods. (C) Tilt schematic for the PBS depicting the four trimeric APC disks in each of the core cylinders. Illustrations and image adapted from Ref. [64].

disk [129], and (iii) the PBs from different PBS disks [127, 130]. The high energy transfer efficiency, although convincing, entertains controversy [131], and thus will benefit from investigations directly at the single molecule level.

Fluorescence microscopy has been a powerful tool in ascertaining information about the spectral properties and heterogeneity of individual proteins and/or complexes not accessible from bulk measurements. Recent single molecule studies of photosynthetic systems has already revealed many new insights [132–139]: (i) In the light-harvesting complex II (LHCII) of the purple bacterium *Rhodopseudomonas acidophila*, bleaching of a single unit of the 18-bacteriochlorophyll assembly halts fluorescence by the entire complex [136]. (ii) For a B-PE molecule, 34 distinct chromophores behave as a single quantum system [137]. (iii) In PEC, more than half of the molecules have significantly different energy transfer efficiencies from their bulk-measured values [138]. (iv) In PBSs of red algae, energy decoupling occurs within the
4.2 Single molecule investigations of energy transfer mechanisms within the phycobilisome

rod under high illumination [139].

To advance the study of energy transfer mechanisms within the PBS, we employ single molecule image deconvolution (SMID) techniques previously developed [34, 52]. According to current models, the high energy transfer efficiency dictates that light is principally emitted by the core’s terminal pigments. From single molecule imaging of wild-type PBSs of *Synechocystis* sp. PCC 6803 and a core mutant lacking PC rods, we fit each frame from a time series using Gaussian analysis; the difference in the respective distributions of measured standard deviations (SDs) suggests that light is emitted at both the core and from the rods with a transfer efficiency less than 95% contrary to prior calculations. This conclusion is further validated from photon counting statistics as well as the observation of multiple, discrete bleaching events in the corresponding fluorescence time traces (see Figs. 4.8 A and 4.9 A) which we associate with individual ‘quenching subunits’ located throughout the intact PBS and comprised of numerous fluorophores behaving as single quantum systems. Localization of these quenching subunits and those specific to the core, implied as being directly correlated to the terminal emitters from analysis of the core mutant alone, is possible with nanometer precision.
4.2 Single molecule investigations of energy transfer mechanisms within the phycobilisome

4.2.2 Methods

4.2.2.1 Characterization of PBS samples

PBSs from a wild-type strain (*Synechocystis* 6803) and a core mutant lacking PC rods were generously provided by Dr. Robert E. Blankenship (Washington University in St. Louis, USA). PBSs were purified following the method described in Ref. [65] and diluted in 20 mM Tris-HCl buffer (pH 8.0) to approximately 0.1 nM. During sample purification, the flexible rods renders the stacked PBPs prone to dissociation. Current methods in quantifying protein size, such as BNPAGE, mass spectroscopy, and TEM, can be invasive and cause further sample degradation. Since intact PBSs are required to address their energy transfer efficiencies in the proposed single molecule experiments, we utilize fluorescence correlation spectroscopy (FCS) as a noninvasive method to accurately measure the sizes and quantities of whole PBS molecules and any dissociated peptides [11, 140].

FCS measurements were performed at the Washington University Fluorescence Correlation Spectroscopy and Confocal Imaging Facility (Department of Biochemistry and Molecular Biophysics, Washington University, USA). Fluorescence from freely diffusing molecules in phosphate buffer was detected as a function of time and an autocorrelation function was obtained from the emitted photon counts

\[ G(\tau) = \frac{1}{N(1 + \frac{4D_\text{m} \tau}{w^2})}, \]  

(4.4)

where \( \tau \) is the detection time, \( N \) is the number of molecules in the detection volume.
4.2 Single molecule investigations of energy transfer mechanisms within the phycobilisome

of radius \( w = 200 \) nm, and \( D_3 \) is the 3D diffusion coefficient of the PBS. If molecules of varying size and type are present in solution, multiple diffusion coefficients can be calculated based on the relative fractions from \( G(\tau) \) as in Eq. (C.1). Analysis of the data (not shown), assuming a typical protein density of 1.38 g/cm\(^3\) for Einstein-Stokes calculations, suggests the PBS samples are intact with an expected size of 60 nm wide.

Another potential problem concerns the attachment of multiple PBSs to the fused-silica surface which may alter the complexes’ organization and subsequently their spectral properties. Single molecule microscopy in combination with excited-state absorption (ESA) spectroscopy was performed for both the wild-type strain and core mutant samples. ESA using excitation wavelengths of 631-707 nm increasing in 11 nm increments revealed absorption peaks, according to photon counting statistics, that were consistent with the spectra from bulk fluorometry measurements (data not shown). We therefore conclude that such surface-altering effects are limited and should not influence the PBS’s energy transfer mechanisms in single molecule studies.

4.2.2.2 Sample preparation and imaging

A PBS solution of 5 \( \mu \)L was sandwiched between manufacturer pre-cleaned fused-silica chips (6W675-575 20C; Hoya Corporation USA, San Jose, CA) and an oxygen-plasma-cleaned coverslip \((2.2 \times 2.2 \text{ cm}^2)\), resulting in a 10.5 \( \mu \)m thick water layer, where isolated PBS molecules were adsorbed to surfaces at low concentration. Single molecule imaging was performed using a Nikon Eclipse TE2000-S inverted microscope (Nikon,
4.2 Single molecule investigations of energy transfer mechanisms within the phycobilisome

Melville, NY) in combination with a Nikon 100X objective (Nikon, 1.49 NA, oil immersion). Samples were excited by prism-type total internal reflection fluorescence (TIRF) microscopy with a circularly polarized 568 nm laser line (I70C-SPECTRUM Argon/Krypton laser; Coherent Inc., Santa Clara, CA) focused on a 40 × 20 µm² region. The incident angle at the fused-silica water interface was 70° with respect to the normal. The 568 nm line was filtered from the multiline laser emission using a polychromatic acousto-optic filter (48062 PCAOM model; NEOS Technologies, Melbourne, FL). The laser excitation was pulsed with illumination intervals of 0.5 ms with excitation intensities of approximately 5.63 kW/cm². Images were captured by an iXon back-illuminated electron multiplying charge coupled device (EMCCD) camera (DV897ECS-BV; Andor Technology, Belfast, Northern Ireland). An additional 2X expansion lens was placed before the EMCCD, producing a pixel size of 79 nm. The excitation filter was 568/20 nm, and the emission filters used included a 525/50 nm narrow band pass filter for SD measurements and a dual filter combining the 525/50 nm filter and a 580 nm long pass filter for localization measurements of quenching subunits (Sec. 4.2.3.1).

4.2.2.3 Data acquisition and selection

PBS movies were obtained by synchronizing the onset of camera exposure with laser illumination. The maximum gain level of the camera was used and the data acquisition rate was 1 MHz pixels/s (≈3.3 frames/s). Single molecule images were checked such that there were no saturations in the intensity profiles. For localization studies,
between an $11 \times 11$ and up to a $17 \times 17$ pixel region, depending on the image’s apparent spot size, centered at the molecule was selected by hand using IMAGEJ (NIH, Bethesda, MD); the center $9 \times 9$ and up to $15 \times 15$ pixels, respectively, containing the point spread function (PSF) were used for subsequent 2D Gaussian fitting, and the peripheral pixels were used for background analysis.

Before analysis, the camera’s intensity count at each pixel in an image was converted into photon count by using the photon-to-camera count conversion factor calibrated the same day of the measurement as described in Ref. [34]. The number of detected photons in an image was obtained by subtracting the total photon count of the background from the total photon count of the image. The PBS intensity profiles were fit to a 2D Gaussian function using a least squares curve fitting algorithm (lsqcurvefit) provided by MATLAB (The Mathworks, Natick, MA):

$$f(x, y) = f_0 \exp \left[ -\frac{(x - x_0)^2}{2s_x^2} - \frac{(y - y_0)^2}{2s_y^2} \right] + \langle b \rangle,$$

where $f_0$ is the amplitude, $s_x$ and $s_y$ are the SDs in the $x$ and $y$ directions, respectively, $x_0$ and $y_0$ are the centroid location of the molecule, and $\langle b \rangle$ is the mean background offset in photons. Additional data analysis was performed in order to measure the relative separation distances between quenching subunits in both the wild-type PBS and core mutant samples.
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4.2.3 Results

According to current models, light is initially harvested at the periphery of PC rods and transferred to reaction centers with an efficiency greater than 95%. It is, therefore, surmised that nearly all light is emitted by the core’s terminal pigments. Under this supposition, the widths of the intensity profiles, corresponding to PBSs from a wild-type strain (*Synechocystis* 6803) and a core mutant which lacks PC rods, should be approximately equal. However, experimental measurements (data not shown) from fitting images of the molecules’ intensity profiles to 2D Gaussian functions [Eq. (1.7)] reveals a difference between the respective SD distributions (and their means). Both PBS samples were imaged simultaneously so as to minimize effects due to defocusing.

4.2.3.1 Localization of quenching subunits

In addition to SD measurements of intensity profiles from both PBS samples, the observation of multiple bleaching events in the corresponding fluorescence time traces, denoted by the arrows in Fig. 4.8 A, indicates that light is emitted by sources other than the core’s terminal pigments. Although photobleaching of individual fluorophores within a complex is common, PBSs from *Synechocystis* 6803 contain 6 (rods) \( \times 18 (\alpha\beta \text{ monomers/rod}) = 108 \) PC monomers and 3 (core cylinders) \( \times 12 (\alpha\beta \text{ monomers/cylinder}) = 36 \) APC monomers for a total of 396 chromophores (within each monomer, chromophores are located on the cystein residues at \( \alpha84, \beta84, \) and \( \beta155, \) the latter being a loop not found in APC), consequently, the expected behavior would be a steady decay in the fluorescence over a long time. Instead, up to nine dis-
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tinct photobleaching events can be discerned (with a mode of five to six events) as well as many erratic traces since bleaching characteristics differ dramatically among PBSs. This suggests complexity and heterogeneity in the spectral properties of PBSs such that several chromophores appear to be grouped into individual quenching subunits associated with each bleaching event.

Quenching subunits are believed to be sources of light emission throughout the PBS. In order to accurately determine these sites of energy leakage, localization is accomplished with nanometer precision using SIMA methods by measuring the molecule’s centroid for each frame of the time series. Since multiple emitters are present, only the final source (frames of constant intensity prior to the last arrow) can be localized relative to the molecule’s average or true center at the start of the time series (frames prior to the first arrow); it is then possible to localize each source successively thereafter in a manner analogous to SHRImP [69]. Figure 4.8 B plots the centroid locations of the PBS molecule having corrected for stage drift by subtracting the measured centroid of an immobilized monomer (PBS molecule displaying only one late bleaching event in the same time series) at each of the specified frames. Although the relative distance of the final quenching subunit can be less than 10 nm with respect to the center, and could therefore be attributed to a terminal emitter, additional data (not shown) reveals larger separations. To eliminate the possibility of aggregates or dissociated PBS peptides, the lifetime photon counts are computed for each time series (with an average of $\approx 4000$ photons) and are expected to be similar; furthermore, SDs considerably larger than those reported in preliminary measurements for
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Figure 4.8: Fluorescence time trace and localization analysis of a PBS molecule from a wild-type strain (*Synechocystis* 6803). (A) Intensity (photons) versus time for a single PBS molecule displaying nine distinct photobleaching events indicated by the arrows. Measurements of the molecule’s centroids from Gaussian analysis of the frames prior to the first arrow and those of constant intensity prior to the last arrow should be associated with the center of the PBS (denoted by the large, purple star) and the relative location of the final quenching subunit (denoted by the small, orange stars scattered throughout the PBS), respectively. (B) Corresponding centroid measurements versus time. The solid lines represent the mean position in each lateral direction with associated error bars. The separation distance is 27.1 ± 9.5 nm.
individual PBSs would be indication of potential aggregates. These selection criteria are used in the subsequent analysis of core mutant samples lacking PC rods, below.

Since many bleaching events are observed for intact PBSs and in order to better differentiate terminal pigments within the core from other sources of emission, localization analysis of core mutant samples was performed following a similar procedure. Figure 4.9 A shows the fluorescence time trace of one PBS molecule lacking PC rods with two discernable bleaching steps of comparable intensities; for time traces that were not indeterminate, the number of bleaching events ranged up to four (with a mode of two events). Figure 4.9 B reports the corresponding distance of the quenching subunit relative to the molecule's center as less than 10 nm and representative of the observed distribution of separations corrected for stage drift (for the last quenching subunit) as shown in Fig. 4.10. The distribution's mean separation distance of 10.1 nm is consistent with our current understanding of the core's organization with respect to the terminal emitters which can be maximally separated by 15 nm. Identification of these terminal pigments from localization analysis and their role in PBS energy transfer is discussed in the following section (Sec. 4.2.4).

4.2.4 Discussion

Current models dictate that light is primarily emitted by the core’s terminal pigments due to reported energy transfer efficiencies greater than 95%. However, SD measurements from Gaussian fitting to intensity profiles of PBSs from a wild-type strain (Synechocystis 6803) and core mutants lacking PC rods suggest light is emitted
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Figure 4.9: Fluorescence time trace and localization analysis of a PBS molecule from a core mutant strain lacking PC rods. (A) Intensity (photons) versus time for the APC core of a single PBS molecule displaying two distinct photobleaching events indicated by the arrows. (B) Corresponding centroid measurements versus time. The solid lines represent the mean position in each lateral direction with associated error bars. The separation distance with and without correcting for stage drift is 12.95 ± 6.16 and 5.66 ± 3.22 nm, respectively.
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Figure 4.10: Distribution of relative separation distances for quenching subunits from core mutant samples. The corresponding Gaussian fit yields a mean separation distance of 10.1 nm and a SD of 5.98 nm; the propagated error from all 29 molecules analyzed is 1.96 nm. Only the separations for the final quenching subunit obtained from each of the molecules are shown such that larger distances associated with aggregates determined according to lifetime photon counts and SD measurements were not included.
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at the core and at the rods by quenching subunits comprised of multiple fluorophores behaving as independent quantum systems. Observation of distinct bleaching events in the fluorescence time traces of these samples which are, on average, comparatively higher in number for intact PBSs as opposed to the core mutants, supports this claim and is further validated by preliminary measurements of the quenching subunits’ separation distances which localizes many of these potential emitters, with nanometer precision, to be outside of the APC core.

Studying a core mutant lacking PC rods reduces the complexity and heterogeneity of the PBS such that we can attribute several bleaching events to specific quenching subunits localized to the core alone. It is possible that the sample has a core complex with differing organization and features as the intact PBS from the wild-type strain; consequently, the measured SD results and fluorescence characteristics may not be reliable but is discounted from the analysis of absorption spectra. Additionally, it may be impossible to say with absolute certainty where these PBS quenching subunits are located with respect to the entire complex and/or to which structural elements they are associated with such as PC disks at the periphery of the rods; nevertheless, several suppositions can be made regarding the terminal pigments, specifically, from localization analysis of core mutant samples.

It is widely accepted that energy is funneled unidirectionally from PC in the rods to long wavelength pigments in the APC core which are in direct contact with the thylakoid membrane [141]. It is further noted, from in vivo fluorescence studies of wild-type cells and mutants, that most emission occurs at the terminal pigments in the
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core rather than by PBPs transferring excitation energy to PSs and is supported by the precise spectral overlap between the emissions from these PBs with the absorption peak of PSII’s reaction center, strongly believed to be recipient of PBS harvested energy and the first protein complex in the light-dependent reactions of photosynthesis [65, 127]. There is a small probability, however, that ‘uphill’ energy flow occurs in the opposite direction given sufficient thermal energy to the local environment [142]. Each of the two lower core cylinders within the PBS actually contains two distinct terminal pigments including the core membrane linker $L_{CM}$, and the PB subunit $\alpha^B$, in the $(\alpha\beta)_2\beta^{18.5}L_{CM}^{99}$ and $(\alpha\beta)_3L_C^{10}$ complexes, respectively [127]. Both pigments were resolved from second derivative spectral analysis discovering two distinct maxima with wavelengths longer than those associated with APC and specifically identifying the longer-wavelength PBS component as the 94 kDa $L_{CM}$ polypeptide which was previously isolated; the two pigments are also independent such that energy from the APC is transferred to them separately with any exchange being prohibited [143].

The $L_{CM}$ linker or ‘anchor polypeptide’ has multiple responsibilities which include: (i) anchoring the APC core to the thylakoid membrane, (ii) involvement with the core’s organization, (iii) directed energy transfer to reaction centers in the thylakoid membrane, and (iv) the ability to modify the spectral properties of neighboring PBPs [126]. It was demonstrated that the $L_{CM}$ alone is sufficient to mediate energy transfer to PSII, while $\alpha^B$ may be associated with PSI [126]. This pathway is favored since the dipole moment between APC and the $L_{CM}$ linker, located in the thylakoid membrane together with PSII which is directly bound to the PBS, is nearly parallel whereas the
αB component has a larger anisotropy suggesting a greater dipole moment thereby lowering its probability for energy transfer [143]. This is consistent with mutagenic studies performed using PBSs with substituted chromophore-binding cystein-186 in the LCM polypeptide and mutants lacking αB which reported a quantum yield that decreased by almost 50% compared to the wild-type strain and no significant change in the fluorescence, respectively [144, 145].

There have been several attempts to elucidate the exact path of excitation energy transfer from the core to, ultimately, the reaction centers in PSs; in vivo studies have shown that sorbitol can disrupt energy transfer between APC and the terminal pigments by osmotic regulation, indicating a potential site [146]. These limited investigations to date, involving fluorescence and spectral analysis, have primarily used whole cells as opposed to individual PBS complexes which are infrequently studied with single molecule imaging [139, 147]. The results of our single molecule experiments have revealed the presence of quenching subunits as possible sites of light emission and energy transfer, specifically within the core. The observations of typically two bleaching events of similarly high intensities (see Fig. 4.9) and, occasionally, fluorescent time traces which include steps at significantly lower intensities by comparison would suggest, from the aforementioned studies, these quenching subunits are associated with the LCM polypeptide and αB subunit, respectively, and can be verified from localization analysis.
4.3 Single molecule investigations of intraflagellar transport and entry mechanisms in *Chlamydomonas*

4.3.1 Introduction

Cilia and flagella are membrane-bound hair-like projections on the surface of numerous eukaryotic and almost all human cells that play an important role as a sensory organelle, being sensitive to the surrounding environment and responding accordingly. While these whip-like appendages are defined by their function rather than their structure, because flagella and cilia are structurally identical, both terms shall be used interchangeably. A flagellum, as shown in Fig. 4.11, is supported by the axoneme which also provides it with an ability to bend due to the orientation of microtubules (MTs) typically arrayed in a ring consisting of nine outer ‘doublet’ MTs in addition to two central MTs. This cytoskeletal network acts as a scaffolding for multiple complexes and allows for binding of motor proteins which carries molecular cargo from the cell.

Figure 4.11: Cross-sectional view of the axoneme of a typical eukaryotic flagellum showing the 9+2 arrangement of MTs. Image taken from Ref. [148].
body to the periphery or flagellar tip and *vice versa*. Accurate sensing and signal transduction is maintained through the concerted actions of flagellar membrane signaling proteins undergoing anterograde (outward) and retrograde (inward) transport by kinesin-2 and cytoplasmic dynein 2/1b, respectively, with a speed of \( \approx 2 \, \mu m/s \), and which is collectively regarded as intraflagellar transport (IFT) [149]. IFT subcomplexes A and B, comprised of many distinct particles, permit continued axonemal growth and are emerging as machinery required in tandem with molecular motors for transport of certain membrane signaling proteins [150].

Malfunctions in the signal reception and transduction pathways are implicated in human as well as other vertebrate diseases, known as ciliopathies defined as genetic disorders of cellular cilia, their anchoring basal bodies, or ciliary function. The growing list of ciliopathies including polycystic kidney disease (PKD) [151] and Bardet-Biedl syndrome (BBS) [152] are likely caused by ciliary dysgenesis and/or IFT dysfunction; consequently, these and other disorders may benefit from single molecule studies of IFT in a model organism such as *Chlamydomonas* [153] in which several flagellar membrane proteins associated with the aforementioned ciliopathies, *i.e.*, Pkd2 and BBSomes, are conserved in humans [150]. *Chlamydomonas* is a genus of green algae approximately 10 \( \mu m \) in size and swims by the synchronized beating of two flagella at its head.

The signal transduction pathway, including the various mechanisms employed by and kinetics of membrane signaling proteins, is not well understood; specifically, questions pertaining to how these proteins (i) enter the flagellum, (ii) travel within the
flagellum according to IFT, and (iii) switch from anterograde to retrograde transport at the flagellar tip, all of which are believed to occur sequentially, demand proper study but only the former is addressed in this work. It is known that membrane signaling proteins are initially synthesized in the cell body and must enter the flagellum near its base; however, the composition of the flagellar membrane is very different from that of the plasma membrane in that a growing number of signaling proteins are found therein [154]. This segregation is due to the presence of two physical barriers: the septin-based membrane located at the flagellar base which prevents free diffusion between the compartments [155] and the ciliary necklace/transition fibers at the basal body which prevents the free diffusion of larger proteins in solution [156]. In order to explain how membrane signaling proteins overcome these diffusion barriers, two different models have been proposed [85, 157]: (i) In Model I (Fig. 4.12 A), post-Golgi vesicles carry the proteins to the basal body, fuse with the membrane near the diffusion barrier, and subsequently enter via lateral diffusion [158]. (ii) In model II (Fig. 4.12 C), which has recently gained support, proteins are bound to BBSome/IFT-particle machinery at the basal body and are carried through the transition fibers into the flagellum.

Investigation of the entry mechanism is accomplished using in vivo SPT experiments focused at the flagellar base such that the two models (see Fig. 4.12 B) predict the membrane signaling protein to undergo either Brownian diffusion or processive motion coinciding with the corresponding molecular motor’s trajectory, respectively. Although fluorescence imaging studies have suggested lateral diffusion of Smo in the
4.3 Single molecule investigations of intraflagellar transport and entry mechanisms in Chlamydomonas

Figure 4.12: Illustrations of two dynamic models for IFT of membrane signaling proteins and their transport machinery divided into three steps (i, ii, and iii) within the flagellum; only two MTs are displayed for clarity. (A) Model I: (i) At the flagellar entry region, a membrane signaling protein such as Pkd2 enters by lateral diffusion. (ii) In the flagellum, the protein becomes associated with an IFT particle and proceeds to undergo anterograde transport along a MT until it dissociates; the bound BBSome may either dissociate as well (case a) or continue traveling to the flagellar tip (case b). (iii) At the flagellar tip, the IFT machinery components dissociate and freely diffuse prior to retrograde transport. (B) Expected displacement versus time trajectories for Pkd2-mCherry (orange), BBSome-GFP (green), and an IFT20 particle (gray) according to Model I (solid lines) and Model II (dashed lines). Straight lines are indicative of processive motion whereas chaotic or random patterns prior to IFT and following dissociation signify Brownian diffusion. (C) Model II: (i) At the flagellar entry region, a membrane signaling protein enters through transition fibers assisted by a BBSome (which is in turn carried by an IFT particle) and proceeds to undergo anterograde transport. (ii) In the flagellum, the protein and its BBSome carrier may dissociate together (case a) or continue traveling to the flagellar tip (case b). (iii) At the flagellar tip, the IFT machinery does not dissociate prior to retrograde transport.
Hedgehog pathway across the membrane diffusion barrier [159], additional analysis stemming from alternative interpretations of the data is required to resolve the ambiguity [85]. To that end, we performed single molecule imaging of Pkd2-GFP and BBSome-GFP proteins, separately, in combination with MSD analysis of individual trajectories to elucidate the correct flagellar entry mechanism. Eventually, simultaneous two-color imaging of Pkd2-mCherry and BBSome-GFP proteins in the flagellar entry region of *Chlamydomonas* should validate the results.

### 4.3.2 Methods

#### 4.3.2.1 Sample preparation and imaging

Pkd2, a homolog of the human protein PKD2, was chosen for the proposed study because: (i) it is an important signaling protein such that mutations in PKD2 are known to cause PKD [160], (ii) it is a transient receptor potential channel that is conserved in humans and *Chlamydomonas* [161], and (iii) it has been labeled with GFP and imaged in *Chlamydomonas* previously. Multiple cell lines independently expressing a GFP tagged BBS4 gene (BBSome-GFP) and Pkd2-GFP were generously provided by Dr. Susan K. Dutcher (Washington University in St. Louis, USA) with cells grown under nutrient-rich conditions.

A 5 µL aliquot of *Chlamydomonas* cells in solution were deposited on manufacturer pre-cleaned fused-silica chips coated with polylysine and sandwiched by an oxygen-plasma-cleaned coverslip (2.2 × 2.2 cm²), resulting in a 10.5 µm thick wa-
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After deposition, the flagella of the isolated cells become attached to the fused-silicate surface while the cell bodies (some of which were adsorbed to surfaces at low concentration) remain oriented in the solution. Single molecule prism-type TIRF microscopy was performed as described in Sec. 4.2.2.2 using a circularly polarized 488 nm laser line. The laser excitation was pulsed with illumination intervals of 1 ms with excitation intensities of approximately 20.75 kW/cm². The excitation filter was 488/10 nm, and the emission filter was 525/50 nm.

The flagella are approximately 200 nm in thickness which is comparable to the penetration depth of the TIRF evanescent field; furthermore, the cell bodies of *Chlamydomonas* cells are oriented away from the fused-silica surface. This creates an ideal setup such that imaging of fluorescent proteins throughout entire flagella can be performed with high signal-to-noise ratios (SNRs) due to the limited interference from the cell bodies which are comparatively brighter.

4.3.2.2 Data acquisition and selection

Movies were obtained by synchronizing the onset of camera exposure with laser illumination; a representative frame from one movie imaging Pkd2-GFP molecules undergoing IFT in a single *Chlamydomonas* cell is shown in Fig. 4.13 B. The maximum gain level of the camera was used and the data acquisition rate was 1 MHz pixels/s (≈3.3 frames/s). Single molecule images were checked to ensure that there were no saturations in the intensity profiles. For SPT studies, kymographs were generated by initially cropping the desired region of interest, containing a single flagellum, from
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the movie, rotating the cropped movie such that the flagellum is aligned perfectly straight, and displaying the frames as a function of time as shown in Fig. 4.14 which corresponds to lower flagellum from the same movie Fig. 4.13 B was taken from. After selecting a fluorescent molecule that appears in multiple neighboring frames of the kymograph, a $17 \times 17$ pixel region centered at the molecule in each frame was selected by hand using ImageJ; the center $15 \times 15$ pixels containing the PSF were used for subsequent 2D Gaussian fitting and localization, while the peripheral pixels were used for background analysis.

Before analysis, the camera’s intensity count at each pixel in an image was converted into photon count by using the photon-to-camera count conversion factor calibrated the same day of the measurement as detailed in Ref. [34]. The number of detected photons in an image was obtained by subtracting the total photon count of the background from the total photon count of the image. Intensity profiles were fit to a 2D Gaussian function as described in Sec. 4.2.2.3 such that $s_x$ and $s_y$ are the SDs in the $x$ and $y$ directions, respectively, $x_0$ and $y_0$ are the centroid location of the molecule, and $\langle b \rangle$ is the mean background offset in photons. Determination of the entry mechanism for proteins subject to either anomalous diffusion or a biased process associated with processive motion is accomplished from MSD analysis of their individual trajectories (i.e., displacement versus time). While imaging of single molecules as opposed to aggregates, which may only appear brighter, is preferred, this consideration should not influence the determination of flagellar entry mechanisms from the SPT studies since centroid measurements are independent of size and can still be

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achieved with nanometer precision; however, the reported diffusion coefficients from single trajectory analysis could be lower than expected.

4.3.3 Results

Individual *Chlamydomonas* cells expressing Pkd2-GFP were imaged *in vivo* under TIRF evanescence. A single frame from movies obtained by differential interference contrast (DIC) and TIRF microscopy are displayed in Figs. 4.13, A and B, respectively, such that IFT of Pkd2-GFP molecules is apparent. The corresponding kymograph for the parent movie of Fig. 4.13 B is shown in Fig. 4.14 for the lower flagellum. Multiple fluorescent proteins are observed as are their trajectories which can be traced throughout the duration of the movie; the majority undergo anterograde transport (*i.e.*, IFT trains) ultimately arriving and waiting at the flagellar tip (*bottom*) while others appear to have dissociated from MTs and are diffusing randomly within the flagellum.

In order to determine the flagellar entry mechanism, Pkd2-GFP molecules near the flagellar base were tracked and their MSDs calculated. Figure 4.15 A displays a cropped portion of the kymograph from Fig. 4.14 in which two trajectories are discernable between 9 and 12 s in length; the top (*blue*) and bottom (*white*) arrows indicate the beginning of a trajectory for a fluorescent molecule within the flagellar entry region (presumably a protein that recently entered) and for one that came into view at approximately the middle of the flagellum or body, respectively. The lateral positions of both molecules were measured from Gaussian analysis and plotted as
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Figure 4.13: DIC and TIRF microscopy images of a *Chlamydomonas* cell expressing Pkd2-GFP. (A) DIC microscopy image of a *Chlamydomonas* cell. (B) Corresponding TIRF microscopy image such that Pkd2-GFP fluorescent proteins are visualized within the flagella. The scale bar is 4 µm.

Figure 4.14: Kymograph for the parent movie of Fig. 4.13 B for the lower flagellum. The movie comprises 300 frames (≈3.3 frames/s) displayed across three rows in which the spatial axis represents time; within each row, Pkd2-GFP fluorescent proteins are observed to undergo anterograde transport from the flagellar base (*top*) to the flagellar tip (*bottom*) and random diffusion within the flagellum. The scale bar is 2 µm.
a function of time in Fig. 4.15 B where the end-to-end displacement within the observation time was \( \approx 1.2 \mu \text{m} \). Since the trajectories aren’t linear with time, it suggests the Pkd2-GFP particles were diffusing within the flagellum as opposed being carried by a BBSome or an IFT particle undergoing anterograde transport.

Figure 4.15: Kymograph and single trajectory MSD analysis for two Pkd2-GFP proteins. (A) Kymograph from frames 51 to 100 of Fig. 4.14 (top row). The top (blue) and bottom (white) arrows indicate the beginning of trajectories for Pkd2-GFP fluorescent proteins in the flagellar entry region and in the middle of the flagellum. The scale bar is 2 \( \mu \text{m} \). (B). Corresponding displacements versus time for the top (blue, solid squares) and bottom (white, open circles) trajectories in A. Both curves were synchronized to start at \( t = 0 \) and were tracked for 9 and 12 s, respectively. (C) Corresponding MSDs as a function of \( n \), according to Eq. (4.5), with associated error bars displayed for data points below \( n_c < 50\% \). Linear fits yielded slopes of 0.74 and 1.12 with \( D_{\text{eff}} = 5.88 \times 10^4 \) and \( 7.12 \times 10^3 \text{ nm}^2/\text{s} \), respectively.

To assess whether the diffusion is Brownian, we employed MSD analysis of single trajectories \([4, 87]\), such that each trajectory is subdivided into displacements \( x \), of varying time intervals \( n \Delta t \),
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\[
\text{MSD}(n, N) = \sum_{i=1}^{N-n} \frac{(x_{i+n} - x_i)^2}{N-n} = 2D_{\text{eff}}n\Delta t + 2\Delta x^2, \quad (4.5)
\]

where \( n \) is an integer, \( \Delta t \) is the shortest time interval between successive observations with \( N \) being the total number of observations, \( D_{\text{eff}} \) is the particle’s effective diffusion coefficient, and \( \Delta x \) is the error associated with each localization measurement. The averages of these squared displacements for the two selected trajectories are plotted in Fig. 4.15C on a log-log scale corrected for the measurement error along with linear fits to the data points below a cutoff value \( n_c \), determined by the fractional uncertainty to \( \text{MSD}(n, N) \) as \( n_c < \sqrt{\frac{2n^2+1}{3n(N-n+1)}} < 50\% \). A particle performed a Brownian walk if its corresponding slope is unity at low \( n \) with \( D_{\text{eff}} \) measured as the intercept along the \( y \)-axis at \( n = 1 \). The fits for the two (top and bottom) trajectories yielded slopes of 0.74 and 1.12 with \( D_{\text{eff}} = 5.88 \times 10^4 \) and \( 7.12 \times 10^3 \) nm\(^2\)/s, respectively; analysis of eight different trajectories from the kymograph of Fig. 4.14 yielded slopes between 0.73 and 1.12 with \( D_{\text{eff}} \) ranging from \( \approx 10^3 \) to \( 5.88 \times 10^4 \) nm\(^2\)/s. These values are in agreement with known diffusion coefficients of various transmembrane proteins in cell membranes [162].

4.3.4 Discussion

Linear fits from MSD analysis of single Pkd2-GFP trajectories suggest membrane signaling proteins enter flagella by lateral diffusion according to Model I. This is consistent with the results of autocorrelation statistics (data not shown) performed on
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the set of localization measurements of proteins first observed within the entry region implying there is no preferred MT association and that flagellar entry is governed by a purely diffusive and random mechanism. Furthermore, the data indicates that diffusion within the flagellum can be anomalous, describing a diffusive process with a non-linear relationship to time, in contrast to typical Brownian diffusion where the MSD \( \langle x^2 \rangle \propto D_{\text{eff}} t^\alpha \), is a linear function of time with \( \alpha = 1 \); specifically, the motion is subdiffusive since many of the reported slopes were less than unity (\( \alpha < 1 \)) which often describes crowded systems such as macromolecular crowding in cells or porous media.

Although the results are suggestive, additional data is required for a more thorough analysis. As previously discussed, it is experimentally challenging to obtain clean trajectories due to flagellar attachment to fused-silica surfaces and *Chlamydomonas* cell body orientations. Moreover, the flagellar basal body is embedded in the cell body for \( \approx 500 \) nm before stemming out [163], and photobleaching of fluorophores or high concentrations of Pkd2-GFP in the flagellum prevents straightforward selection of individual trajectories. A promising solution is to rely on single-image measurements of protein diffusion coefficients for when trajectories are not sufficiently long for accurate study [53]; if the diffusion coefficient is within the acceptable range of reported values, above, we can reasonably conclude that proteins were not undergoing IFT. However, the inability to distinguish between aggregates and single molecules, albeit rare and which usually require long fluorescent time traces to observe an individual bleaching event, may lead to diffusion coefficients lower than expected. Consequently,
we also should not discount the possibility that Models I and II are not mutually ex-
clusive and that we may have only tracked diffusing Pkd2-GFP molecules by chance
or proteins that prematurely dissociated from MTs after being carried by transition
fibers near the flagellar entry region.

It is currently known that Pkd2 is carried by IFT trains in *Chlamydomonas* flag-
ella [161] but the BBSome, which acts as a coat protein for ciliary trafficking of
various molecular cargo, may also be responsible or influence the membrane signaling
protein’s intraflagellar dynamics [164]. To confirm whether Pkd2 is carried by IFT
or BBSome/IFT particle machinery throughout the flagellum, simultaneous studies
of Pkd2-mCherry and BBSome-GFP fluorescent proteins using two-color imaging is
warranted. Analysis of such overlapping trajectories which if revealed as processive
(see Fig. 4.12 B) would lend support for Model II with regards to the flagellar entry
mechanism and/or IFT within the flagellum.
Chapter 5

Summary and Conclusion

*It can scarcely be denied that the supreme goal of all theory is to make the irreducible basic elements as simple and as few as possible without having to surrender the adequate representation of a single datum of experience.* -Albert Einstein, “On the Method of Theoretical Physics”, the Herbert Spencer Lecture (1933)

The advent of single molecule imaging has enabled researchers to elucidate many of the underlying, complex processes that occur within the cell; furthermore, the use of total internal reflection fluorescence (TIRF) microscopy minimizes the contribution of background noise and permits selective visualization of single fluorescent proteins in a restricted region of the specimen, such as within the plasma membrane for *in vivo* studies, near the glass-water interface. However, localization and single particle tracking (SPT) of these molecules is limited by the Rayleigh criterion to distances greater than $\sim210$ nm as is the ability to report the precisions of such measure-
ments in an accurate and timely fashion which is not currently possible due to the tradeoff between spatial and temporal resolution. The objective of this dissertation was to address these challenges by using analytical techniques to improve the temporal resolution associated with three-dimensional (3D) localization of proteins and determination of their corresponding diffusion coefficients without sacrificing spatial resolution and information. Additionally, we have applied these methods, which are collectively referred to as single image molecular analysis (SIMA), to study several biological systems of interest.

In Chap. 2, we introduce the concept of single molecule image deconvolution (SMID) in order to advance ‘super-localization’ methods with improved temporal resolution utilizing an analytical expression derived for the error associated with measuring the standard deviation (SD) of a fluorescent molecule’s intensity profile given a single image. This equation incorporates experimental parameters such as the number of detected photons $N$, the finite pixel size of the camera, $a$, and various contributions of the background noise and has been validated through simulations and experimental studies. While the SD is used to infer a particle’s axial position, the corresponding localization precision is usually determined via repeated imaging or image stacks. The failure to report the positional accuracy on faster timescales significantly limits the application and efficiency of SPT; consequently, we have demonstrated that a comparable error can be achieved using the aforementioned equation lifting the temporal resolution to the typical exposure time of milliseconds. We have also shown that the SD and its error are able to discriminate between a single molecule
and a complex (i.e., dimer) as well as measure the separation distances in the latter case.

Furthermore, it was observed that since the intensity profile of a freely-diffusing fluorophore imaged for a finite exposure time undergoes motion-induced blurring, a relationship between the molecule’s diffusion coefficient and the measured SD can be established, as discussed in Chap. 3. Although SPT is capable of measuring the diffusion coefficient while preserving spatial information between imaging times, the temporal resolution is limited to seconds such that it cannot address fast diffusive processes. By directly imaging fluorescent proteins and studying the widths of their intensity profiles from single images, we determine their respective diffusion coefficients to known error at submillisecond exposure times. We verified our technique using eGFP molecules and demonstrated that fast tracking studies can be performed without loss in precision.

Finally, Chap. 4 is dedicated to applying the previously described analytical techniques to study various biological systems including: (i) protein-DNA interactions, (ii) energy transfer within the phycobilisome (PBS), and (iii) intraflagellar transport (IFT) and entry mechanisms in Chlamydomonas. We conducted Monte Carlo simulations in order to calculate accurate sliding diffusion coefficients of DNA-binding proteins from previous single molecule experiments; moreover, the simulations revealed the proper time scales future studies need in order to assess hopping (and sliding) kinetics. Single molecule investigations of PBSs suggest that light is emitted variedly throughout the complex contrary to the belief that it occurs solely at
the terminal pigments with high quantum efficiency. This is supported by additional
evidence indicating the presence of discrete ‘quenching subunits’, each comprising
multiple fluorophores acting as a single quantum system, and which can be localized
with SIMA methods. It is surmised that within the core, these quenching subunits are
directly correlated with the terminal pigments chiefly responsible for energy transfer
to reaction centers in cyanobacteria. Lastly, we performed in vivo studies of Pkd2-
GFP, a signaling protein known to undergo IFT in Chlamydomonas and is associated
with motility and signal transduction along the membrane. Mean square displace-
ment analysis of individual particle trajectories implies anomalous diffusion within
the flagellar entry region near the membrane, consistent with one of two competing
models; the range of our reported diffusion coefficients for Pkd2 are in agreement
with values of other transmembrane proteins in cell membranes.

In summary, the studies performed in this dissertation constitute an improvement
in temporal resolution over existing methods by employing single-image measure-
ments. Furthermore, we have demonstrated that the measured SD and its corre-
sponding error, via an analytical expression derived in Chap. 2, can determine the
diffusion coefficient of a freely-diffusing protein in addition to other novel applica-
tions. Use of these techniques to address biological problems of interest represents
ongoing research which will be continued in future studies that are envisioned.
Appendices

You are young, and your bitter recollections have time to change themselves into sweet remembrances. -Alexandre Dumas, The Three Musketeers (1844)
Appendix A

Precision analysis of standard deviation measurements

A.1 Photon-to-camera count conversion effects

When an EMCCD camera is used in imaging single fluorescent molecules, the detected pixel reading is in camera counts. In converting from camera counts to photon counts, an additional variance in $\sigma_{i,\text{photon}}$ appears. Below we derive the uncertainty in photon counts, $\sigma_i$, to use in place of $\sigma_{i,\text{photon}}$ in Eq. (2.3) for experiments where EMCCD camera count conversions are involved.

An EMCCD camera amplifies the detected photons by converting each photon to a distribution of photoelectrons through many multiplication stages. At the final stage, one photon yields a distribution of camera counts (equivalent to the last stage
A.1 Photon-to-camera count conversion effects

photoelectron counts) with a distribution function \( f(n^*) \) [165],

\[
f(n^*) = \frac{1}{M} \exp\left(-\frac{n^*}{M}\right),
\]

where \( n^* \) is the camera counts in the distribution and \( M \) is the photon multiplication factor of the camera. Here we use \( ^* \) to denote camera counts in order to differentiate from photon counts. The \( n^* \) distribution has a mean of \( M \) and a variance of \( M^2 \).

At pixel \( i \), the PSF photon count distribution is described by a Poisson distribution with the variance being equal to the mean. Each photon arriving at a pixel contributes two terms to the pixel’s camera count variance: the mean photon shot noise variance \( M^2 \) (variance of a single photon, \( i.e. \), one, multiplied by the square of the multiplication factor), and the photon-to-camera count conversion variance \( M^2 \).

The total camera count variance contributed by one photon is \( 2M^2 \); therefore, a mean of \( N_i \) photons yields a camera count variance of \( 2N_iM^2 \). This is in agreement with the expression in Ref. [166] where the variance in camera counts \( \sigma^2_{\text{out,camera}} \), is related to the variance in photon counts \( \sigma^2_{\text{in,photon}} \), by an excess noise factor \( F^2 \),

\[
F^2 = \frac{1}{M^2} \frac{\sigma^2_{\text{out,camera}}}{\sigma^2_{\text{in,photon}}} \approx 2
\]

for EMCCD cameras with a large number of multiplication stages.

Fluorescence from buffer, diffusing molecules in the solution, and camera counts associated with electronic readout and thermal noise constitute the total background
A.2 Derivation of $\langle (\Delta s)^2 \rangle$

Photon count at pixel $i$, with a variance of $\sigma_i^2$ and a mean of $\langle b \rangle$. The total background variance in camera counts is the sum of the background count variance $\sigma_b^2 M^2$, and the variance introduced by the average number of background photons, $\langle b \rangle$, each with a variance of $M^2$: $(\sigma_b^2 + \langle b \rangle) M^2$.

Summing the PSF and the background contributions, the total camera count variance at pixel $i$ is

$$\sigma^2_i = 2N_i M^2 + (\sigma_b^2 + \langle b \rangle) M^2.$$  \hfill (A.3)

When expressed in photon counts,

$$\sigma_i^2 = \sigma_i^*^2 / M^2 = 2N_i + \sigma_b^2 + \langle b \rangle.$$  \hfill (A.4)

### A.2 Derivation of $\langle (\Delta s)^2 \rangle$

Here we present the complete derivation of Eq. (2.3). We first obtain a probability distribution function for $y_i$. At large $N$ of a few hundred photons, the $y_i$ probability distribution function at each of the center nine pixels of the PSF is a Gaussian, while at the peripheral pixels, the $y_i$ probability distribution function is better approximated by a Poisson with a low mean. Here we assume that our $N$ is significantly larger than 100 photons and the $y_i$ probability distribution functions for all PSF pixels are Gaussian functions

$$f_{y_i} = \frac{1}{\sqrt{2\pi}\sigma_i} \exp \left( -\frac{\Delta y_i^2}{2\sigma_i^2} \right).$$  \hfill (A.5)
A.2 Derivation of $\langle (\Delta s)^2 \rangle$

where $\Delta y_i = N_i(x_0, s_0) - y_i$ and $\sigma_i^2$ is $\sigma_{i,\text{photon}}^2$ as in Eq. (2.1). For Gaussian distributed $y_i$, we have

$$\langle \Delta y_i \rangle = 0,$$  \hspace{1cm} (A.6a)

$$\langle (\Delta y_i)^2 \rangle = \sigma_i^2.$$  \hspace{1cm} (A.6b)

Starting from Eq. (2.1) and taking a derivative with respect to $s$,

$$\frac{d\chi^2(s)}{ds} = \sum \frac{d}{ds} (y_i - N_i)^2 \sigma_i^2 = \sum \frac{2(y_i - N_i)(y_i - N_i)' \sigma_i^2 - (y_i - N_i)^2 \cdot 2\sigma_i \sigma_i'}{\sigma_i^4}. \hspace{1cm} (A.7)$$

Setting the above equation to zero, we find

$$\sum \frac{2(y_i - N_i)(y_i - N_i)'}{\sigma_i^2} = \sum \frac{(y_i - N_i)^2 \cdot 2\sigma_i \sigma_i'}{\sigma_i^4}. \hspace{1cm} (A.8)$$

We can simplify Eq. (A.8) using the following terms:

$$y_i - N_i(s) = y_i - (N_i(s_0) + N_i' \Delta s) = -\Delta y_i - N_i' \Delta s, \hspace{1cm} (A.9a)$$

$$(y_i - N_i)' = -N_i', \hspace{1cm} (A.9b)$$

$$\sigma_i^2 = 2N_i(s) + \sigma_b^2 + \langle b \rangle = 2(N_i(s_0) + N_i' \Delta s) + \sigma_b^2 + \langle b \rangle, \hspace{1cm} (A.9c)$$

$$2\sigma_i \sigma_i' = 2N_i'. \hspace{1cm} (A.9d)$$
Inserting Eqs. (A.9a)-(A.9d) into Eq. (A.8), we obtain

\[
\sum_i -2 \left( \Delta y_i + N_i' \Delta s \right) (-N_i') \sigma_i^2 = \sum_i \frac{(\Delta y_i + N_i' \Delta s)^2 \cdot 2N_i'}{\sigma_i^4} \\
\approx \sum_i \frac{(\Delta y_i^2 + 2\Delta y_i N_i' \Delta s) \cdot 2N_i'}{\sigma_i^4}.
\]  

(A.10)

Moving \(\Delta s\) to the left-hand side,

\[
\Delta s \sum_i \left( \frac{N_i'^2}{\sigma_i^2} - \frac{2\Delta y_i N_i'^2}{\sigma_i^4} \right) = \sum_i \left( \frac{\Delta y_i^2 N_i'}{\sigma_i^4} - \frac{\Delta y_i N_i'}{\sigma_i^2} \right)
\]

(A.11)

\[
\Delta s = -\frac{\sum_i \frac{\Delta y_i N_i'}{\sigma_i^2} \left( 1 - \frac{\Delta y_i}{\sigma_i} \right)}{\sum_i \frac{N_i'^2}{\sigma_i^2} \left( 1 - 2\frac{\Delta y_i}{\sigma_i} \right)}.
\]

(A.12)

Neglecting the \(\Delta y_i/\sigma_i^2\) term, we get

\[
\Delta s \approx -\frac{\sum_i \frac{\Delta y_i N_i'}{\sigma_i^2}}{\sum_i \frac{N_i'^2}{\sigma_i^2}}.
\]

(A.13)

We now take the mean square of Eq. (A.13). Note that the average is meant to apply to \(y_i\) only, so we have

\[
\langle (\Delta s)^2 \rangle = \frac{\sum_i \frac{\Delta y_i N_i'}{\sigma_i^2} \sum_j \frac{\Delta y_j N_j'}{\sigma_j^2}}{\left( \sum_i \frac{N_i'^2}{\sigma_i^2} \right)^2} = \frac{\sum_{i,j} \frac{\Delta y_i \Delta y_j N_i' N_j'}{\sigma_i^2 \sigma_j^2}}{\left( \sum_i \frac{N_i'^2}{\sigma_i^2} \right)^2}.
\]

(A.14)

For two different pixels, their distributions are independent, so \(\langle \Delta y_i \Delta y_j \rangle = \delta_{ij} \langle (\Delta y_i)^2 \rangle = \sigma_i^2\) [see Eq. (A.6b)]. This gives us Eq. (2.3).
A.3 Extending $\Delta s_{rms}$ to 2D

In 2D, the expected counts at pixel $i, j$ is given by

$$N_{i,j} = \frac{Na^2}{2\pi s_x s_y} \exp \left[ -\frac{(ia)^2}{2s_x^2} - \frac{(ja)^2}{2s_y^2} \right],$$  \hspace{1cm} (A.15)

where we assume that the PSF is centered at zero. Taking the derivative of $N_i$ with respect to $s_x$ and evaluating at $s_{0x}$,

$$\langle (\Delta s_x^2) \rangle = \frac{1}{\sum_i \left( \frac{dN_i}{ds_x} \right)^2 \sigma_i^2}. \hspace{1cm} (A.16)$$

Next, we approximate the summation by an integral where $i$ and $j$ are continuous from negative to positive infinity. In the high photon count limit, $\langle (\Delta s_x^2) \rangle = \frac{s_{0x}^2}{N}$ after taking the photon-to-camera count conversion variance into consideration, while in the high background noise limit, $\langle (\Delta s_x^2) \rangle = \frac{16\pi \alpha_{0x} \alpha_{0y} (\sigma_b^2 + \langle b \rangle)}{3\alpha^2 N^2}$. Adding these terms together in quadrature and accounting for the pixelation effect, we obtain $\Delta s_{x,rms}$ [Eq. (2.9)].
Appendix B

Single-image axial localization analysis

B.1 Derivation of $\Delta z$

It has been previously shown that the measured SD of an intensity profile, $s_i$, scales with the axial position as

$$s_i(z) = s_f\sqrt{1 + \left(\frac{z}{d}\right)^2 + B \left(\frac{z}{d}\right)^4},$$

(2.12)

where $s_f = \sqrt{s_f^2(z = 0) + \frac{a^2}{12}}$ is the PBS SD at focus including the pixelation effect, $d$ is the imaging depth of the microscope, and $B$ is a higher order fitting parameter to correct for the refractive index mismatch effect and the non-ideality of an imaging system. The precision of axial localization measurements can be obtained from propagat-
B.1 Derivation of $\Delta z$

ing the uncertainty associated with measuring $s_i$, $\Delta s_i$, specifically $\Delta z = (\frac{\partial s_i}{\partial z})^{-1} \Delta s_i$.

Dropping the higher order $B$ term which is often needed for prism-type TIRF microscopy, the error is found to be

$$\Delta z = \frac{\Delta s_i d^2}{s_f} \sqrt{\frac{1}{z^2} + \frac{1}{d^2}}$$

$$= \Delta s_i d \sqrt{\frac{1}{s^2 - s_f^2} + \frac{1}{s_f^2}}. \quad (B.1)$$

To calculate the complete axial localization error (including $B$), the derivative of Eq. (2.12) with respect to $z$ is required,

$$\frac{\partial s_i}{\partial z} = \frac{s_f z (d^2 + 2Bz^2)}{d^4 \left[1 + \left(\frac{z}{d}\right)^2 + B \left(\frac{z}{d}\right)^4\right]^{1/2}}. \quad (B.2)$$
Since Eq. (B.2) is dependent on \( z \), we can solve for it by inverting Eq. (2.12),

\[
z = \pm \frac{d}{\sqrt{2Bsf}} \left\{ \left[ s_f^2 + 4B (s_i - s_f) (s_i + s_f) \right]^{1/2} - s_f \right\}^{1/2}
\]

\[
= \pm \frac{d}{\sqrt{2Bs_f}} \left\{ s_f \left[ (1 - 4B) + 4B \left( \frac{s_i}{s_f} \right)^2 \right]^{1/2} - s_f \right\}^{1/2}
\]

\[
= \pm \frac{d}{\sqrt{2B}} \left\{ 2 \left[ B \left( \frac{s_i}{s_f} \right)^2 - B + \frac{1}{4} \right]^{1/2} - 1 \right\}^{1/2}
\]

\[
= \pm \frac{d}{B^{1/4}} \left\{ \left( \frac{s_i}{s_f} \right)^2 - 1 + \frac{1}{4B} \right\}^{1/2} - \frac{1}{2\sqrt{B}} \right\}^{1/2}
\]

\[
= \pm \frac{d}{B^{1/4}} \left( C - \frac{1}{2\sqrt{B}} \right)^{1/2},
\]

where \( C = \sqrt{\left( \frac{s_i}{s_f} \right)^2 - 1 + \frac{1}{4B}} \). Using Eqs. (2.13) and (B.2), the axial localization error \( \Delta z \), can be calculated with its complete derivation provided and broken up as follows:

\[
\Delta z_{num} = d^4 \left( 1 + \frac{d^2}{d^2 \sqrt{B}} \left\{ \left( \frac{s_i}{s_f} \right)^2 - 1 + \frac{1}{4B} \right\}^{1/2} - \frac{1}{2\sqrt{B}} \right)
\]

\[
+ \frac{Bd^4}{d^4 B} \left\{ \left( \frac{s_i}{s_f} \right)^2 - 1 + \frac{1}{4B} \right\}^{1/2} - \frac{1}{2\sqrt{B}} \right\}^{2} \Delta s_i
\]

\[
= d^4 \left( \frac{s_i}{s_f} \right) \Delta s_i,
\]
\[
\Delta z_{\text{den}} = \frac{s_f d}{B^{1/4}} \left\{ \left[ \left( \frac{s_i}{s_f} \right)^2 - 1 + \frac{1}{4B} \right]^{1/2} - \frac{1}{2\sqrt{B}} \right\}^{1/2} \\
\quad \cdot \left( d^2 + \frac{2d^2 B}{\sqrt{B}} \left\{ \left[ \left( \frac{s}{s_f} \right)^2 - 1 + \frac{1}{4B} \right]^{1/2} - \frac{1}{2\sqrt{B}} \right\} \right) \\
= \frac{s_f d^3}{B^{1/4}} \left( C - \frac{1}{2\sqrt{B}} \right)^{1/2} \left[ 1 + 2\sqrt{B} \left( C - \frac{1}{2\sqrt{B}} \right) \right] \\
= 2s_f d^3 B^{1/4} \left( C - \frac{1}{2\sqrt{B}} \right)^{1/2} .
\] (B.4)

Combining the above equations, we obtain the axial localization error [Eq. (2.14)],

\[
\Delta z = \frac{d^4 \left( \frac{s_i}{s_f} \right) \Delta s_i}{2s_f d^3 B^{1/4} \left( C - \frac{1}{2\sqrt{B}} \right)^{1/2}} = \frac{ds_i \Delta s_i}{2s_f^2 B^{1/4} \left( C - \frac{1}{2\sqrt{B}} \right)^{1/2}} \\
= \frac{ds_i \Delta s_i}{\sqrt{2}s_f^2 C \left( 2C \sqrt{B} - 1 \right)^{1/2}} .
\] (2.14)
Appendix C

Single-image diffusion coefficient measurements

C.1 Starting locations of imaged diffusing eGFP molecules

In order to correctly simulate diffusing eGFP molecules near the fused-silica surface, the axial starting positions are needed. We obtained the eGFP diffusion starting position PDFs at different exposure times by simulating a fluorophore’s emitted photon distributions for a range of starting positions.

At each exposure time, 1000 diffusion trajectories along the axial dimension were simulated starting from the glass-water interface to an extended distance in water (\(z = 0\) to \(117 + 3\sqrt{2D_{3D}t}\) nm measuring from the reflective fused-silica surface at focus, where \(z = 0\)). The simulations included the triplet-state effect, such that the number
of photons emitted at each step was the mean of a Poisson photon distribution. At each starting position, we obtained the ratio of the number of photons emitted within the penetration depth ($z_d = 117$ nm) to all emitted photons for a simulated trajectory. The mean ratios for all 1000 trajectories are plotted in Fig. C.1. The exposure times shown are 0.3, 0.7, and 1 ms, and truncated Gaussian functions are fit to the distributions. The fitted SD values of the starting position PDFs increase with the exposure time as $SD(t) = 1.538 \times 10^5 t + 122.4$ nm. For $t = 0.3$ ms, Fig. C.1 A shows that most molecules we observed experimentally should start within 200 nm of the surface.

Figure C.1: Simulation results for the diffusing eGFPs’ starting location distribution near the fused-silica-water interface at exposure times of 0.3, 0.7, and 1 ms (A, B, and C) and their corresponding fits.
C.2 Pathway distribution functions in the axial and lateral directions

C.2.1 PWDF$_z$

In order to obtain the eGFP PWDF in the axial direction, diffusion trajectories along the axial dimension were simulated for all exposure times using the starting position PDFs described above and a reflective fused-silica surface at $z = 0$. Figure C.2 A shows 9 representative simulated PWDF$_z$s for the 0.6 ms exposure time. Because a Gaussian function reliably fits to many of the individual PWDFs (84.5% of the data with $R^2 > 0.7$), it should satisfactorily describe PWDF$_z$ mathematically. Figure C.2 B shows the cumulative SD distribution of the fitted PWDF$_z$s and the corresponding Gaussian fit with a mean SD = $\sqrt{A_z \cdot 2D_{3D} t} = 75.8$ nm, yielding $A_z = 0.054$. $A_z$ remains constant for other exposure times with a mean value of 0.052. Figure C.2 C shows the mean value $z_0$, distribution of the fitted PWDF$_z$s and the corresponding Gaussian fit with a mean $z_0 = 142.7$ nm. The inset to Fig. C.2 C shows that $\langle z_0 \rangle$ increases with $t$ as $\langle z_0 \rangle = \sqrt{0.27 D_{3D} t} + 25.5$ nm. For each exposure time, 1000 trajectories were simulated to obtain the results.

C.2.2 PWDF$_x$

In the lateral directions, we numerically calculate $g(x, y)$ of a freely diffusing eGFP particle by simulations. Figure C.3 A shows 9 random PWDF$_x$s the 0.6 ms exposure
Figure C.2: Study of the eGFP axial PWDF's. (A) Nine randomly selected PWDF's at $t = 0.6$ ms and Gaussian fits to the unimodal distributions with $R^2 > 0.7$. (B) Cumulative SD distribution for all fitted PWDF's and its corresponding Gaussian fit with a mean of 75.8 nm. (C) Cumulative mean $z_0$, distribution for all fitted PWDF's and its corresponding Gaussian fit. Inset shows $\langle z_0 \rangle$ increases with $D_{3D}t$. 
time. Six of the nine PWDFs appear unimodal and can be fitted to a Gaussian function with $R^2 > 0.8$. Figure C.3B shows the cummulative SD distribution of PWDFs, combining the Gaussian fitted and statistical SDs for the unimodal and bimodal PWDFs, respectively, with a mean SD = $\sqrt{A_x^2 \cdot 2D_{3D}t} = 96.8$ nm, yielding $A_x = 0.0882$. We found $A_x$ to be insensitive to exposure times below 1 ms with a mean value of 0.0926. Figure C.3C shows that when the 9 PWDFs in Fig. C.3A are convolved with single-eGFP PSFs at focus with $s_f = 108.2$ nm, all convolved PWDFs fit well to a Gaussian function, and the mean of the SD distribution is 147.1 nm. Although not all PWDFs are unimodal, the Gaussian assumption is nevertheless a valid approximation, particularly when convolved with the respective PSF.

C.3 Refractive index mismatch corrections to eGFP intensity profiles

When a fluorophore in water is imaged through a glass coverslip using a high NA oil immersion objective, the refractive index mismatch between the two media modifies the corresponding intensity profile: (i) due to Snell’s law of refraction, such that the true axial position of the molecule (measured from the glass coverslip-water interface) is deeper than the molecule’s apparent axial position (defined as the depth in water where the PSF’s amplitude is maximal) [30], and (ii) due to spherical aberrations, such that if the focus is at the apparent position of the molecule (defined as $z = 0$), the plot of the intensity profile’s SD as a function of $z$ is asymmetric and is
Figure C.3: Study of the eGFP lateral PWDFs and their convolution with PSFs. 
(A) Nine random eGFP PWDFs at $t = 0.6$ ms and Gaussian fits to the unimodal distributions with $R^2 > 0.8$. 
(B) Cumulative SD distribution of 1000 PWDFs and its corresponding Gaussian fit. 
(C) The nine PWDFs in A convolved with eGFP PSFs at focus with $s_f = 108.2$ nm. 
(D) Cumulative SD distribution for 1000 PWDFs convolved with eGFP PSFs at focus and its corresponding Gaussian fit.
C.3 Refractive index mismatch corrections to eGFP intensity profiles

exacerbated when imaging deeper into solution [57, 167] as is the case of the eGFP molecules which were adsorbed on or diffusing near the fused-silica surface in TBE buffer 10.5 µm from the coverslip-water interface. Figure C.4 A shows the geometry of our prism-type TIRF imaging setup, where the direction of $z$ is positive towards the glass coverslip, opposite to that of Ref. [168]. We used fused-silica for its low background noise and thus high SNR as opposed to glass coverslips associated with objective-type TIRF.

To accurately calculate the SD (and $D_{3D}$) of defocused intensity profiles using Eq. (3.8), theoretical calculations and experimental measurements of eGFP molecules adsorbed on the fused-silica surface were made as a function of the defocusing distance. In calculations, we obtained the defocused PSF using diffraction integral analysis in Ref. [168], which has been used by other groups primarily for determining the true axial position of the imaged fluorophores [30, 78]. We assume the final defocused PSF to be the average of four emission polarizations of the fluorophore at $0$, $\pi/4$, $\pi/2$, and $3\pi/4$, with homogeneous light intensity at the emission’s spherical wavefront before reaching the objective. Figures C.4, B and C show the $s_x$ values and amplitudes of the calculated defocused PSFs (in blue) for our imaging setup with a numerical aperture of $NA = 1.49$, water’s refractive index of $n_1 = 1.34$, glass’ refractive index of $n_2 = 1.515$, and an emission wavelength of $\lambda = 525$ nm.

Experimental measurements of eGFP molecules adsorbed on the fused-silica surface were performed using a focus drive (H122; Prior Scientific Inc., Rockland, MA) moving one-way in 100 nm increments through the focus. The mean $s_x$ values
Figure C.4: Theoretical and experimentally measured $s_x$ values and amplitudes versus $z$ for our imaging setup. (A) Our imaging setup and illustration of fluorescent emission in water at the glass-water interface 10.5 µm away from the coverslip-water interface. Dashed lines trace the emission from the true axial position of the fluorophore, and solid lines trace the emission from the fluorophore’s apparent axial position. The letter ‘W’ labels the emission wavefront before reaching the objective. (B) Theoretical (blue circles) and experimentally measured (red squares) $s_x$ values of eGFP PSFs and (C) amplitudes versus the defocusing distance $z$, plots. Lines are piece-wise fits to the experimental measurements. The focus is the minimum of the $s_x$ versus $z$ curve (same for $s_y$).
C.3 Refractive index mismatch corrections to eGFP intensity profiles

and normalized amplitudes of the defocused intensity profiles are plotted in Figs. C.4, B and C (in red). Piecewise fits were employed such that for \( z < 100 \, \text{nm} \),

\[
s_x = s_f \sqrt{1 + \left( \frac{z}{990.3} \right)^2}
\]

where \( s_f = 108.2 \, \text{nm} \) is the minimum of the curve designating the focus (\( z = 0 \)), while for \( z > 100 \, \text{nm} \), a linear fit yields a slope of 0.73. The shape of the \( s_x \) versus \( z \) curve is consistent with that predicted according to theoretical calculations, albeit the values are 30 nm, on average, higher and likely due to a combination of the pixelation effect of the camera, the finite bandwidth of the emission filter, the inhomogeneity of the molecule’s emission polarization, and the imperfection of current single molecule imaging systems. The experimental results are, however, in agreement with reported values in recent publications using similar imaging setups [3, 4, 34, 43]. As a result, the measured PSF amplitudes are lower than their corresponding theoretical values. Piecewise fits to the defocused PSF amplitudes were employed, again, such that for \( z < 150 \, \text{nm} \),

\[
C(z) = \frac{1}{1 + \left( \frac{z + 140.7}{726.7} \right)^2},
\]

while for \( z > 150 \, \text{nm} \),

\[
C(z) = \frac{1}{1 + \left( \frac{z + 389.4}{389.4} \right)^2}.
\]

The peak of \( C(z) \) does not coincide with the focus, rather it is shifted to \( z = -140 \, \text{nm} \). The experimental eGFP functions were used for theoretical and simulated diffusing eGFP SD studies in this article. These results were used in theoretical calculations [Eq. (3.8)] and simulations for intensity profiles of diffusing eGFP molecules.
C.4 Mean emitted photon counts at each simulation step

The number of photons emitted at each simulation step are initially drawn randomly from a Poisson distribution with a mean of $A e^{-z/z_d}$, where $e^{-z/z_d}$ describes the decaying evanescent light intensity with a penetration depth of $z_d \approx 117$ nm calculated for our incident angle of $70^\circ$ [10], and $A$ is a scaling factor that accounts for the quantum efficiency of eGFP molecules. After photon-to-camera count conversion ($M = 1$) which introduces additional variance [34], and incorporating triplet-state statistics, the modified photon count distributions were compared to those obtained from experimental measurements. $A$ was estimated from the mean of the expected photon count distribution for the same simulated diffusion trajectory and chosen when a good match between the two distributions was achieved. Figure C.5 compares the experimental and simulated photon count distributions at an exposure time of 0.6 ms with $A = 0.80$ ($A$ remains approximately constant for all exposure times with a mean of 0.86; data not shown). $A$ is not included in Eq. (3.8) since it does not influence the $s_x$ calculations for defocused PSFs. The corresponding experimental and simulated $s_x$ distributions of diffusing eGFP’s intensity profiles at an exposure time of 0.6 ms are shown in Fig. C.6.
C.4 Mean emitted photon counts at each simulation step

Figure C.5: Comparison of experimental *(black)* and simulated *(empty)* photon count distributions at an exposure time of 0.6 ms. Their respective Gaussian fits in solid and dashed lines are in good agreement.

Figure C.6: Comparison of experimental *(green)* and simulated *(red)* $s_x$ distributions of diffusing eGFP’s intensity profiles at an exposure time of 0.6 ms.
C.5 FCS determination of eGFP’s diffusion coefficient

In order to independently verify our experimentally-determined mean SD values of eGFP intensity profiles [Eq. (3.9) and subsequently $D_{3D}$ values] from theoretical calculations and simulations, we performed FCS measurements of eGFP molecules (at the Washington University Fluorescence Correlation Spectroscopy and Confocal Imaging Facility in the Department of Biochemistry and Molecular Biophysics).

Fluorescence from freely diffusing eGFP molecules at 3 nM concentration in 0.5X TBE buffer (pH 8.0) was measured. An autocorrelation function was used to obtain diffusion parameters for eGFP [169],

$$G(\tau) = \frac{1}{N \left(1 + \frac{\tau}{\tau_d}\right) \sqrt{1 + \frac{\tau}{s^2 \tau_d}}} \left[1 - F + F \exp \left(-\frac{\tau}{\tau_k}\right) \right] + 1, \quad \text{(C.1)}$$

where $\tau$ is the detection time, $N$ is the number of molecules in the detection radius $w$, $s$ is the structure parameter of the excitation beam focal region (the ratio of the beam radius in $z$ to the beam radius in $x$ and $y$), $\tau_d = \frac{w^2}{4D_{3D}w}$ is the molecule’s diffusion time in the imaging area, $F$ is the fraction of molecules in the triplet state, and $\tau_k$ is the triplet-state lifetime.

The excitation wavelength for the FCS measurement was 488 nm, and the emission photons went through a 505-550 nm filter. The excitation power was 76.4 kW/cm$^2$, which was comparable to our excitation power of 37.5 kW/cm$^2$ in the diffusing eGFP.
C.5 FCS determination of eGFP’s diffusion coefficient

studies. We used Alexa 488 with a known diffusion coefficient $D_{3D} = 4.35 \times 10^8 \text{ nm}^2/\text{s}$ [13] for calibration and obtained $w \approx 250 \text{ nm}$ with $\tau_d = 35.6 \mu\text{s}$. Figure C.7 shows $G(\tau)$ versus $\tau$ for eGFP studies with $\tau_d = 174.8 \mu\text{s}$. Assuming a Gaussian detection volume, a one-component fit yields $F = 12.7$, $\tau_k = 3 \mu\text{s}$, and $s = 10$. Using $\tau_d = \frac{w^2}{4D_{3D}}$, we obtained eGFP’s $D_{3D} = 8.86 \times 10^7 \text{ nm}^2/\text{s}$ which is consistent with reported values [13].

Figure C.7: Diffusing eGFP FCS autocorrelation plot. The black curve is a fit to the raw data (red dots).
C.6 Exposure time limits for $D_{3D}$ measurements using Eq. (3.10)

In Fig. 3.4, the calculated SD starts to deviate from the experimental and simulation results at an exposure time of 0.8 ms. This suggests the existence of an upper bound to the exposure time for our eGFP studies. The reason for this discrepancy provides a means for determining an appropriate exposure time in future single molecule studies.

The PWDFs in Fig. C.3 A show both unimodal and bimodal distributions. When convolved with PSFs at short exposure times, the resulting intensity profiles are unimodal and fit well to Gaussian functions; however, as the exposure time increases, so does the fraction of bimodal PWDFs, which after convolution with PSFs, can result in bimodal intensity profiles due to increasing peak separations. Since Eq. (3.1) assumes an intensity profile from the convolution of the PSF and PWDF is well approximated by a Gaussian and can be fit to accordingly, a deviation arises between the analytical and the experimental $s_x$ values which increases with exposure time.

When two identical fluorophores are separated by more than the diffraction limit, the combined intensity profile appears bimodal [52]. Conversely, when multiple fluorophores are localized within a diffraction-limited spot such that their separations are less than the system’s diffraction limit, as is the case for unimodal PWDFs, the combined intensity profile appears unimodal, and can be fitted to a Gaussian function. For the case of bimodal PWDFs, two clusters of fluorophores have a peak separation
C.6 Exposure time limits for $D_{3D}$ measurements using Eq. (3.10)

distance that is approximately twice the SD of the molecule’s location distribution, or $2\sqrt{A_x \cdot 2D_{3D}}t$ of the PWDF$_x$s. When this distance is greater than the diffraction limit of 217 nm, the convolved intensity profiles begin to appear bimodal; the threshold is reached at an exposure time of $t \approx 0.8$ ms, where the PWDF$_x$s’ mean SD is 113.6 nm.

Accordingly, an upper bound to the exposure time for future $D_{3D}$ measurements using Eq. (3.10) would require $2\sqrt{A_x \cdot 2D_{3D}}t$ to be less than the diffraction limit separation given the imaging system’s emission wavelength and NA.

For a particle of unknown $D_{3D}$, the exposure time can be scanned until diffusing particle images are noticeably larger than those of stationary particles, while remaining unimodal. In this range of exposure times, Eq. (3.10), originally for eGFP, can be used to measure $D_{3D}$ where $s'_f$ is calculated from integration that depends on $D_{3D}t$. Because $A_{x,y} \cdot 2D_{3D}t$ varies only with $D_{3D}t$, although $D_{3D}$ of the diffusing particle may be different from that of eGFP, $D_{3D}t$ values can be equivalent at appropriate exposure times. At such exposure times, Eq. (3.10) remains valid and should determine the particle’s unknown $D_{3D}$. 
Appendix D

Protein sliding and hopping kinetics on DNA

D.1 Protein-DNA collision simulations

In simulations, proteins perform a 3D Brownian walk such that each step along any dimension is drawn from a Gaussian distribution with a mean of zero and standard deviation of $\delta = \sqrt{2D_{3D}\tau}$

\[
\begin{align*}
x_{n+1} &= x_n + \sqrt{2D_{3D}\tau} \cdot \mathcal{N}(0,1), \quad \text{(D.1a)} \\
y_{n+1} &= y_n + \sqrt{2D_{3D}\tau} \cdot \mathcal{N}(0,1), \quad \text{(D.1b)} \\
z_{n+1} &= z_n + \sqrt{2D_{3D}\tau} \cdot \mathcal{N}(0,1), \quad \text{(D.1c)}
\end{align*}
\]
D.1 Protein-DNA collision simulations

where \( n + 1 \) signifies the proposed location from the previous one, \( n \). Figures 4.2 and D.1 A demonstrate how the proposed coordinate of a protein, which moves ballistically between consecutive steps, yields a trajectory that passes through DNA; while the protein’s proposed coordinate is far beyond the DNA’s radius in these examples, a collision should have occurred (as would if the proposed coordinate been within the DNA’s radius). Consequently, we need to determine if and where a protein collides with DNA assuming 100% probability for association. According to Fig. D.1 A, if \( d_1 \leq r_{\text{DNA}} + r_{\text{protein}} \) and \( 0 \leq d_2 \leq c \), a protein collides with DNA along its trajectory; moreover, if \( a \leq r_{\text{DNA}} + r_{\text{protein}} \), the protein’s proposed coordinate is located within the DNA’s radius. This analytical result is derived from simple trigonometry in which the triangle formed by the vertices associated with the protein’s previous and proposed locations as well as the DNA’s center has sides of length

\[
\begin{align*}
a &= \sqrt{x_{n+1}^2 + z_{n+1}^2} , \\
b &= \sqrt{x_n^2 + z_n^2} , \\
c &= \sqrt{(x_{n+1} - x_n)^2 + (z_{n+1} - z_n)^2} ,
\end{align*}
\]  

(D.2a)  

(D.2b)  

(D.2c)

with angles (opposite \( a \) and \( b \)) respectively,
\[ \alpha = \arccos \left( \frac{c^2 + b^2 - a^2}{2bc} \right), \]  

\text{(D.3a)}

\[ \beta = \arccos \left( \frac{a^2 + c^2 - b^2}{2ac} \right). \]  

\text{(D.3b)}

d_1 \text{ is the length of the perpendicular drawn from the center of the DNA to the line connecting the last two protein locations, while } d_2 \text{ corresponds to the distance between where } d_1 \text{ intersects this line and the protein’s proposed coordinate:}

\[ d_2 = a \cdot \cos(\beta) = b \cdot \cos(\alpha) \]

\[ = \frac{a^2 + c^2 - b^2}{2c}, \]  

\text{(D.4a)}

\[ d_1 = \sqrt{a^2 - d_2^2} = \sqrt{b^2 - d_2^2}. \]  

\text{(D.4b)}

Although unnecessary for the hopping simulations conducted in Sec. 4.1, the absolute position of where the protein would bind to DNA according to its point of impact can be calculated such that

\[ \theta_1 = \arctan \left( \frac{z_{n+1} - z_n}{x_{n+1} - x_n} \right), \]  

\text{(D.5a)}

\[ \theta_2 = \arctan \left( \frac{z_n}{x_n} \right). \]  

\text{(D.5b)}

From Fig. D.1 \textit{B}, \theta_1 \text{ and } \theta \text{ (used in the following collision scenarios) are defined}
Figure D.1: Detailed schematics for protein-DNA association. (A) The proposed trajectory of a protein (*black circles*) passing through DNA (*orange circle*) forming a triangle with sides $a$, $b$, and $c$, and angles opposite them of $\alpha$, $\beta$, and $\gamma$, respectively. A collision is likely to occur along this trajectory at the position connecting the radii of DNA and the protein (*gray circle*). $d_1$ is the length of the perpendicular drawn from the center of the DNA to the line defining the proposed trajectory, while $d_2$ corresponds to the distance between where $d_1$ intersects this line and the protein’s proposed coordinate. (B) Detailed view of the collision in A with the angles colored red (between the horizontal line and the line parallel to the protein’s proposed trajectory both passing through the DNA’s center) and blue (between the line connecting the radii and the parallel line) corresponding to $\theta_1$ and $\theta$, respectively.
D.1 Protein-DNA collision simulations

by the angles colored red and blue, respectively. Evaluating each of the possible collisions a protein can make with DNA using a left-handed coordinate system (such that Fig. D.1 illustrates the sixth scenario, below, in which the protein’s trajectory crosses DNA from above with a $\oplus, \oplus$ direction), the points of collision (neglecting displacement along DNA in the $y$ direction) are:
\[ \text{sign}(x_{n+1} - x_n) = \oplus \]

\[ \text{sign}(z_{n+1} - z_n) = \ominus \]

(1) \( \theta_1 > \theta_2 \) (below)

\[ x = -(r_{\text{DNA}} + r_{\text{protein}}) \cdot \cos(|\theta_1 - \theta|) \]

\[ z = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \text{sign}(\theta_1 - \theta) \cdot \sin(|\theta_1 - \theta|) \]

(2) \( \theta_1 < \theta_2 \) (above)

\[ x = -(r_{\text{DNA}} + r_{\text{protein}}) \cdot \cos(\theta_1 + \theta) \]

\[ z = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \sin(\theta_1 + \theta) \]

\[ \text{sign}(x_{n+1} - x_n) = \ominus \]

\[ \text{sign}(z_{n+1} - z_n) = \oplus \]

(3) \( \theta_1 < \theta_2 \) (below)

\[ x = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \cos(\theta_1 + \theta) \]

\[ z = -(r_{\text{DNA}} + r_{\text{protein}}) \cdot \sin(\theta_1 + \theta) \]

(4) \( \theta_1 > \theta_2 \) (above)

\[ x = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \cos(|\theta - \theta_1|) \]

\[ z = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \text{sign}(\theta - \theta_1) \cdot \sin(|\theta - \theta_1|) \]
\[\text{sign}(x_{n+1} - x_n) = \oplus\]

\[\text{sign}(z_{n+1} - z_n) = \oplus\]

(5) \(\theta_1 < \theta_2 \) (below)

\[x = -(r_{\text{DNA}} + r_{\text{protein}}) \cdot \cos (\theta_1 + \theta)\]

\[z = -(r_{\text{DNA}} + r_{\text{protein}}) \cdot \sin (\theta_1 + \theta)\]

(6) \(\theta_1 > \theta_2 \) (above)

\[x = -(r_{\text{DNA}} + r_{\text{protein}}) \cdot \cos (|\theta - \theta_1|)\]

\[z = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \text{sign}(\theta - \theta_1) \cdot \sin (|\theta - \theta_1|)\]

\[\text{sign}(x_{n+1} - x_n) = \ominus\]

\[\text{sign}(z_{n+1} - z_n) = \ominus\]

(7) \(\theta_1 > \theta_2 \) (below)

\[x = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \cos (|\theta_1 - \theta|)\]

\[z = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \text{sign}(\theta_1 - \theta) \cdot \sin (|\theta_1 - \theta|)\]

(8) \(\theta_1 < \theta_2 \) (above)

\[x = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \cos (\theta_1 + \theta)\]

\[z = (r_{\text{DNA}} + r_{\text{protein}}) \cdot \sin (\theta_1 + \theta)\]  

(D.6)
D.2 Derivation of ‘diffusion to capture’ probability

In order to quantitatively determine the probability and mean time to capture for a particle performing a random walk near a particular body, we must first solve the diffusion equation. Provided there is a source and adsorber for particles at different points within a system, the net diffusion flux $J$, is proportional to the slope of the concentration $C$, and measures how many particles with diffusion coefficient $D$, flow through a small area as a function of time. In three dimensions, Fick’s first law of diffusion is

$$\vec{J} = -D \nabla C.$$  \hfill (D.7)

Since the number of particles is conserved and therefore obeys the continuity equation, Fick’s second law (the diffusion equation) follows

$$\frac{\partial C}{\partial t} = D \nabla^2 C = D \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) = D \frac{\partial C}{\partial r} + r \frac{\partial^2 C}{\partial r^2},$$  \hfill (D.8)

where it has been rewritten assuming the problem is spherically symmetric with a radial flux $J_r$.

The actual problem maintains that proteins in an infinitely large bath diffuse randomly near a short segment of DNA which can be approximated as a rigid cylinder. However, attempts to solve Eq. (D.8) under these conditions only yield quasi steady-state and time-dependent solutions for diffusion to a cylinder of finite and infinite
D.2 Derivation of ‘diffusion to capture’ probability

length, respectively [170–172]. Consequently, we impose the following boundary conditions such that particles released near a spherical adsorber of radius \(a\) can either be adsorbed by DNA or escape (to infinity) by diffusing to a spherical shell adsorber of radius \(c\):

\[
C(r = a) = 0, \quad (D.9a)
\]
\[
C(r = \infty) = C_0, \quad (D.9b)
\]
\[
\nabla^2 C = 0, \quad (D.9c)
\]

which necessitates that Eq. (D.8) goes to zero,

\[
\Rightarrow \frac{D}{r} \left( \frac{\partial C}{\partial r} + r \frac{\partial^2 C}{\partial r^2} \right) = 0. \quad (D.10)
\]

The problem is illustrated in Fig. D.2 in which particles are released at a spherical shell source of radius \(b\). The general solution (with the imposed boundary conditions above), is of the form
\[ C(r) = C_1 \ln(r) + C_2, \quad (D.11) \]
\[ C(a) = C_1 \ln(a) + C_2 = 0, \quad (D.12a) \]
\[ C(b) = C_1 \ln(b) + C_2 = C_0, \quad (D.12b) \]
\[ C(c) = C_1 \ln(c) + C_2 = 0, \quad (D.12c) \]

such that the particular solution to Eq. (D.8) is

\[
C(r) = \frac{C_0 \ln(r)}{\ln(b/a)} - \frac{C_0 \ln(a)}{\ln(b/a)},
\]

\[
= \begin{cases} 
\frac{C_0 \ln(r/a)}{\ln(b/a)} & a \leq r \leq b \\
\frac{C_0 \ln(r/c)}{\ln(b/c)} & b \leq r \leq c.
\end{cases}
\]  

\[ (D.13) \]

The radial flux is

\[
J_r(r) = -D \frac{\partial C}{\partial r} = \begin{cases} 
\frac{C_0 D}{r \ln(b/a)} & a \leq r \leq b \\
\frac{C_0 D}{r \ln(b/c)} & b \leq r \leq c.
\end{cases}
\]

\[ (D.14) \]

Therefore, the diffusion rates from the spherical shell source are
D.2 Derivation of ‘diffusion to capture’ probability

Figure D.2: Schematic for particles released at a spherical shell source at \( r = b \) and permitted to move inward to be adsorbed at \( r = a \) with a rate \( I_{\text{in}} \) or move outward to be adsorbed at \( r = c \) with a rate \( I_{\text{out}} \).

\[
I_{\text{in}} = (2\pi hr) = -J_{r,\text{in}} = \frac{2\pi hC_0D}{\ln(b/a)}, \quad \text{(D.15a)}
\]

\[
I_{\text{out}} = (2\pi hr) = J_{r,\text{out}} = \frac{2\pi hC_0D}{\ln(b/c)} = \frac{2\pi hC_0D}{\ln(c/b)}. \quad \text{(D.15b)}
\]

The probability of capture that a particle released at \( r = b \) will be adsorbed by DNA at \( r = a \) is simply the ratio

\[
P = \frac{I_{\text{in}}}{I_{\text{in}} + I_{\text{out}}} = \frac{\frac{1}{\ln(b/a)}}{\frac{1}{\ln(b/a)} + \frac{1}{\ln(c/b)}} = \frac{\ln(c/b)}{\ln(c/b) + \ln(b/a)} = \frac{\ln(c/b)}{\ln(c/a)}. \quad \text{(D.16)}
\]

In the limit \( b \to a \), the probability is one, that is all particles beginning on the surface
of the cylinder will return after a sufficiently long random walk; a steady state or limit
does not exist for $b \to \infty$, as discussed earlier, but should ultimately approach one
as well. If DNA is not treated as a perfect adsorber and a particle released at $r = b$
could collide with DNA, dissociate and return again, the mean number of round trips
before it eventually escapes to infinity can also be calculated as

$$\langle n \rangle = \frac{p}{1 - p} = \frac{\ln(c/b)}{\ln(c/a) - \ln(c/b)} = \frac{\ln(c/b)}{\ln(b/a)}. \quad (D.17)$$

For our simulations, proteins were initially located at the protein-center-to-DNA-
center distance of $R = r_{\text{DNA}} + r_{\text{protein}} + \Delta r$ and permitted to dissociate from DNA by
taking a single Brownian step. Given the geometry of the cylinder (with $r^2 = y^2 + z^2$)
and that proteins are not considered point-like particles, the effective release distance
$b$, is derived by averaging over all possible, valid steps from DNA, as follows

$$b^2 = \int_0^\infty (\sqrt{2} \delta)^2 + a^2 - 2a(\sqrt{2} \delta) \cos(\theta + \pi/2) d\theta, \quad (D.18)$$

or

$$b^2 = \langle x^2 \rangle + \langle (a + z)^2 \rangle \quad (D.19a)$$

$$= \langle x^2 \rangle + a^2 + 2a \langle z \rangle + \langle z^2 \rangle$$

$$= \frac{1}{\sqrt{2\pi\delta}} \int_0^\infty x^2 e^{-x^2/2\delta^2} dx + a^2 + 2a \frac{2}{\sqrt{2\pi\delta}} \int_0^\infty z e^{-z^2/2\delta^2} dz + \frac{2}{\sqrt{2\pi\delta}} \int_0^\infty z^2 e^{-z^2/2\delta^2} dz$$

$$= 2\delta^2 + a^2 + 2a\delta \sqrt{\frac{2}{\pi}}, \quad (D.19b)$$

where $\delta$ is the protein’s step size and $a = R$. 

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