SINGLE-MOLECULE SPECTROELECTROCHEMICAL INVESTIGATION OF FREELY DIFFUSING REDOX-ACTIVE FLUOROPHORES IN ZERO-MODE WAVEGUIDES

A Dissertation

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by Dane A. Grismer

Paul W. Bohn, Director

Graduate Program in Chemical and Biomolecular Engineering

Notre Dame, Indiana

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Single-molecule studies with optical techniques have become instrumental in
shedding light upon the heterogeneities in molecular property distributions, which are
averaged out in ensemble measurements. Gaining insights into the nature and
magnitude of such heterogeneities in molecular systems, particularly when they are
subject to anisotropic chemical, optical, electrical, or other perturbations, has important
implications in the design of targeted detection schemes. Strategies that enable
detection and manipulation of subpopulations of molecules have the potential to
contribute in transformational ways to lab-on-a-chip (LOC) analytical systems and point-
of-care (POC) diagnostic devices.

The work in this dissertation aims to provide a novel approach for the
electrochemical manipulation of redox-active fluorophores coupled with spectroscopic
measurements. The design, construction, and utilization of a fluorescence correlation
spectroscopy (FCS) optical system for the measurement of freely diffusing single-
molecules form the basis for more advanced studies. Piezoelectric stages for nanometer spatial resolution and advanced acquisition hardware for sub-nanosecond temporal resolution are implemented. The fabrication of Au-clad zero-mode waveguide (ZMW) arrays by focused ion beam (FIB) milling is described. Simulations of the decay of the optical field within ZMWs support the basis for single-molecule experiments.

Spectroelectrochemical measurements of the redox-active fluorophore, flavin mononucleotide (FMN), were performed at single-molecule concentrations in a bulk solution above an indium tin oxide (ITO) electrode and in the approximately 200 zL observation volumes of individual electrochemical ZMWs (E-ZMWs). Fluorescence analogs of standard electrochemical experiments were implemented. These are fluorescence emission under static control and chronofluorometry as analogs to amperometry, and cyclic potential sweep fluorometry as an analog to cyclic voltammetry. A long-lived semiquinone state of FMN was stabilized and observed in a ZMW.

The work in this dissertation represents the first single-molecule spectroelectrochemistry investigations in an E-ZMW nanophotonic structure. Means of improving the performance of the FCS optical system and expanding its capabilities are explored. Experimental modifications, device design variations, and other possible redox-active chemical systems are discussed.
For my lovely bride, Kathleen,

my joyous son, John,

and wonderful parents, Timothy and Matrell
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“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”

J. R. R. Tolkien, *The Hobbit*

Tolkien’s words speak to the commonality that I believe runs through the type of adventure he wove into *The Hobbit* and the task of a researcher. Our carefully planned experiments often lead us to stumble upon something different, and usually far more interesting, than what we first sought. The excitement of finding the unknown and undiscovered is perhaps the most treasured reward that the adventure of research provides.

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1.1 Single-Molecule Studies as Keys to Molecular Understanding

The 2014 Nobel Prize in Chemistry was awarded to Eric Betzig, Stefan W. Hell, and William E. Moerner “for the development of super-resolved fluorescence microscopy.”\(^1\) The contributions made separately by Betzig and Moerner were to single-molecule microscopy. Though distinctly different techniques than the methods described in this work, the appreciation that single-molecule studies have garnered illustrates their current and future importance in optically-based measurements. As has been revealed in many single-molecule studies, heterogeneities in molecular property distributions are averaged out in ensemble measurements.\(^2\)-\(^4\) Thus, it is important to reveal the nature and magnitude of such heterogeneities, in order to more accurately describe the behavior of molecular systems, particularly, when they are subject to anisotropic chemical, optical, electrical, or other perturbations. Additionally, learning how to detect and manipulate subpopulations of molecules has the potential to contribute in transformational ways to lab-on-a-chip (LOC) analytical systems and point-of-care (POC) diagnostic devices.
1.2 Fluorescence Correlation Spectroscopy (FCS)

1.2.1 Inception and Early Studies

Magde, Elson, and Webb laid the conceptual framework for fluorescence correlation spectroscopy (FCS) as a tool to measure fluctuations in a system about a chemical equilibrium.\(^5^6\) They first demonstrated the technique with the reversible binding of ethidium bromide to DNA.\(^5^7\) The binding creates a strongly fluorescent complex, which they allowed to freely diffuse into and out of a small open boundary excitation volume. A photodetector recorded fluorescence intensity fluctuations, mathematically expressed as,

\[
\delta F(t) = F(t) - \langle F(t) \rangle
\]  

(1.1)

where \(F(t)\) is the instantaneous fluorescence intensity and angle brackets \(<>\) denote a time average. \(\delta F(t)\) then corresponds to intensity fluctuations in the detected fluorescence. The average fluorescence intensity, \(\langle F(t) \rangle\), is expressed as,

\[
\langle F(t) \rangle = \frac{1}{T} \int_{0}^{T} F(t) dt
\]  

(1.2)

The normalized autocorrelation function, \(G(\tau)\), can be calculated from Equations 1.1 and 1.2,\(^8\)

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

(1.3)
and then used to extract diffusion and kinetic parameters. The simplest system that can be addressed is a bulk fluid with a homogeneous concentration profile with no convection, containing a low, typically pM-nM concentration of a single brightly fluorescent and photostable species. From this simple system, the concept can be expanded to more complex chemical systems and to the measurement of flow velocity and reaction rates. The basis for all of these methods is that the same rate coefficients underlie the rates of decay of both microscopic and macroscopic deviations from equilibrium. Analyzing the rates of decay from spontaneous concentration fluctuations enables determination of diffusion coefficients through fitting of the autocorrelation function, $G_D(\tau)$, with,

$$G_D(\tau) = \frac{1}{\langle N \rangle} \left[ 1 + \frac{\tau}{\tau_D} \right]^{-1} \left[ 1 + S^2 \left( \frac{\tau}{\tau_D} \right) \right]^{-\frac{1}{2}} \tag{1.4}$$

where $\langle N \rangle$ is the average number of fluorescent molecules in the probe volume, $\tau$ is the delay time, $\tau_D$ is the diffusion time through the probe volume, and $S$ is the ratio of the $1/e^2$ beam half-height, $z_0$, to the $1/e^2$ beam waist radius, $r_0$, at the diffraction-limited focal point. The diffusion coefficient, $D$, can be calculated from the diffusion time by,

$$D = \frac{r_0^2}{n \tau_D} \tag{1.5}$$

where $n$ is the number of dimensional degrees of freedom, or it can be used as a fitting parameter in an alternate form of the autocorrelation function by substitution of Equation 1.5 into Equation 1.4,
\[ G_D(\tau) = \frac{1}{\langle N \rangle} \left( 1 + \frac{4D\tau}{r_0^2} \right)^{-1} \left( 1 + \frac{4D\tau}{z_0^2} \right)^{-1/2} \]  

(1.6)

where \( r_0 \) and \( z_0 \) are the same quantities describing the beam shape in the ratio \( S \) in Equation 1.4.\(^9\) Accurate analysis requires full calibration of the system and determination of the equipment parameters, including the beam shape, beam size, and excitation power. Using a small probe volume and dilute fluorescent molecule concentrations in the single-molecule range is key to achieving a signal-to-noise ratio sufficient to be able to detect single events and distinguish them from the background signal.\(^6\) Poisson statistics estimate the distribution, \( P(n; \lambda) \), of target molecules in the probe volume at any given time using,

\[ P(n; \lambda) = \frac{e^{-\lambda} \lambda^n}{n!} \]  

(1.7)

where \( n \) is an integer number of molecules and \( \lambda \) is the average number of molecules in the probe volume.\(^9\) For a 0.1 fl probe volume and a concentration of 100 pM, the probabilities of 0, 1, or 2 molecules occupying the probe volume at any given time are 0.994, 0.006, and 0.000, respectively.

1.2.2 Resurgence and Technique Refinement

FCS did not become widely available until two decades after the initial demonstration, at which time a wide range of improvements greatly decreased the necessary acquisition time and increased the experimental success rate.\(^10\) Four important improvements were critical in improving the overall success rate. Confocal
optics improved signal-to-noise ratio by reducing the observation probe volume to less than 1 fl. \(^{11}\) Hardware correlators with improved temporal resolution enabled faster processes to be accurately observed. Multiple correlation channels allowed for cross-correlation of two detectors to mitigate the contribution of detector noise, such as afterpulsing. \(^{10}\) Avalanche photodiodes provided greater quantum efficiency, outpacing advances in photomultipliers. \(^{12}\) Using a confocal fluorescence microscope, Nie and colleagues were able to detect individual fluorescein molecules in an aqueous medium for the first time. \(^{13}\) Instrument precision was increased again by using shorter integration times and accounting for boundary recrossings in and out of the probe volume. \(^{14}\)

Not only does FCS allow for confirmation of diffusion coefficients determined by other methods, it enables determination of diffusion coefficients in systems not accessible to testing by other means. Commercial FCS systems make it a popular and accessible technique in a variety of fields, particularly biology. \(^{15}\) An advantage of the technique is that measurements of reactions involving non-photolytic species can be made, as long as, a change in fluorescence or diffusion properties occurs as a result of the reaction. \(^{6,16}\) FCS has been used to measure or characterize concentrations, diffusion coefficients, reaction rates, hydrodynamic radii, molecular binding, and molecular structure, \(^{8}\) as well as, flow rates. \(^{17}\)
1.2.3 Application to Confinement

Because FCS can be applied to different physical geometries, chemical systems, and experimental conditions, maintaining self-consistency from experimental design through acquisition to analysis is essential. The primary problems arise in analysis, where the correct equation must be used to fit the experimental autocorrelation curve, otherwise, using inapplicable models for free diffusion can lead to significant deviations.\(^{18-19}\) The standard model for bulk solutions and non-confining geometries, such as wells and microchannels, is a three-dimensional model which accounts for free diffusion in all directions. If the channel is confined in one direction to a length-scale commensurate with a physical scaling length of the chemical system being observed, a two-dimensional model is necessary to account for diffusion being constrained to two-axes.\(^{20-21}\) Similarly, for molecules confined to movement along one direction, a one-dimensional model would be necessary. Thus, confinement has the effect of creating anomalous subdiffusion, in which the observed diffusion coefficient is less than its bulk value.\(^{22}\)

1.3 Zero-Mode Waveguides (ZMWs) and Single-Molecule Studies

1.3.1 Theory and Inception

Zero-mode waveguides (ZMWs) provide confinement conditions that are ideally suited for the study of single-molecules. ZMWs were developed and first used by Webb and coworkers to study biologically-relevant processes near their native concentrations.\(^{23}\) Traditional single-molecule spectroscopy techniques couple reduction
in the observation volume with sample dilution to produce single occupancy conditions. Confocal techniques, including modern implementation of FCS (vide supra, Section 1.2.1), achieve this through a tightly-focused diffraction-limited excitation volume on the order of $10^{-16}$ L and require pico- to nanomolar concentrations for single occupancy conditions. Total internal reflection produces an evanescent wave that restricts excitation to approximately 100 nm beyond the glass-solution interface, increasing the signal-to-background ratio and producing single occupancy conditions at a higher concentration. ZMWs reduce the observation volume by several additional orders of magnitude. Physical nanopore volumes of $10^{-19} - 10^{-18}$ are readily attainable, providing excellent spatial confinement. They also provide optical confinement as they do not support far-field propagating modes when the wavelength is greater than a critical wavelength $\lambda_c \approx 1.7d$, where $d$ is the pore diameter. Under these conditions, the optical field is evanescent within the pore, with a magnitude that decays exponentially along the longitudinal (i.e., $z$) axis of the pore,

$$I(z) = e^{-z/\Lambda}$$  \hspace{1cm} (1.8)

where $\Lambda$ is the decay constant and $z$ is the depth into the pore. The decay constant is defined as,

$$\frac{1}{\Lambda} = 2\left[\left(\frac{1}{\lambda_c}\right)^2 + \left(\frac{1}{\lambda_m}\right)^2\right]^{1/2}$$  \hspace{1cm} (1.9)

where $\lambda_m$ is the wavelength in the ZMW medium. For a cylindrical pore, the decay of the evanescent field produces an effective observation volume, $V_{eff}$, given by,
\[ V_{\text{eff}} = \frac{\pi d^2 \Lambda}{24} \]

For example, a pore with a 100 nm diameter, filled with water, and excited at 458 nm produces \( V_{\text{eff}} \approx 100 \text{ zL} \). This corresponds to a relatively high \( <N> = 1 \) concentration of 17 μM. As an additional comparison of the techniques, diffraction-limited FCS can study only a small fraction of enzymes at concentrations corresponding to their Michaelis constant, \( K_m \), values.\(^{27}\) Coupled with a ZMW, FCS can be extended to study the majority of recorded enzymes at their \( K_m \) values because single-molecule occupancy can be achieved at much higher bulk concentrations. In their seminal work, Webb and coworkers used this ability of ZMWs to study the enzymatic synthesis of DNA by DNA polymerase at micromolar concentrations.\(^{23}\) FCS curves showed that the time molecules spent within the nanopore was approximately an order of magnitude less than in a diffraction-limited volume. Despite this short residence time, the higher concentration of molecules provided sufficient signal-to-noise ratio to obtain high quality correlation data.

1.3.2 Applications of ZMWs

The use of ZMWs has been extended to single enzyme kinetics,\(^{28}\) protein interactions,\(^{29}\) membrane diffusion,\(^{30}\) and other single-molecule studies.\(^{31}\) The short skin depth of Al makes it the standard metal of choice for the cladding layer in ZMWs, as it effectively attenuates optical field propagation. Bohn and coworkers have taken a different approach with the utilization of Au cladding.\(^{32-34}\) Due to its large ideally
polarizable region, it enables electrochemical manipulations inside of the pores (*vide infra*, Section 1.4). A wide range of thiol-based surface chemistries are available to functionalize molecules to the Au surface to enable immobilization-based experiments. The chemical orthogonality between Au and SiO$_2$, the common substrate supporting the Au, allows for selective passivation of the Au surface.\textsuperscript{35} Altogether, Au serves in a multifunctional capacity as the optical cladding layer of the ZMW and as the working electrode of an electrochemical cell. The fullest implementation of ZMWs, thus far, is Pacific Bioscience’s Single Molecule Real Time (SMRT) DNA sequencing system, first commercialized in 2011.\textsuperscript{36} Marketed as third-generation DNA sequencing, massive multiplexing of ZMWs with DNA polymerase immobilized on the bottom of the pores enables sequencing by observation of fluorescently labeled DNA bases.

1.4 Spectroelectrochemistry

The first single-molecule electrochemical experiments were performed with electrochemiluminescence\textsuperscript{37} and redox cycling.\textsuperscript{38-39} Since that time, interest in investigating single-molecule electrochemistry has increased,\textsuperscript{40} particularly by means of coupling fluorescence and electrochemistry. Early applications of coupled total internal reflectance fluorescence (TIRF) microscopy and electrochemistry targeted absorption at chemical interfaces and the development of new biosensors.\textsuperscript{41-44} In these experiments, redox-sensitive optical properties of the molecule—either the absorption cross-section, the fluorescence quantum efficiency, or both—were spectroscopically measured to probe electron transfer events. The utility of coupling electrochemical measurements
and fluorescence is evidenced by recent studies of redox cycling, catalyst screening, and diffusion zone imaging.\textsuperscript{45-48} A common feature of coupled electrochemical-luminescence experiments is that a current measurement, with the attendant large background, is replaced by the detection of luminescent photons, in principle, a zero background measurement.

Barbara, Bard, and coworkers were the first to demonstrate single-molecule spectroelectrochemistry (SMSEC) as an approach to study electron transfer (ET).\textsuperscript{49} They were motivated by the challenge of characterizing the innate heterogeneity of interfacial boundaries in a way in which the unique distributions of various thermodynamic and kinetic processes could be captured rather than just relying on an ensemble average. They used an indium tin oxide (ITO) electrode for redox cycling of an organic polymer. In this and subsequent studies,\textsuperscript{50-52} distinct differences in single-molecule fluorescence dynamics were observed at $E_{appl}$ values above and below the equilibrium potential, $E_{eq}$, thereby, firmly establishing fluorescence spectroscopy as a tool to monitor the dynamics of single electron transfer events.

SMSEC was used to study electron transfer dynamics of cresyl violet by Ackerman and coworkers.\textsuperscript{53} Using an ITO-coated coverslip as the working electrode, a single-molecule concentration solution of freely diffusing cresyl violet was observed by scanning fluorescence microscopy. They showed that fluorescence intensity time traces correspond directly with the applied potential, with few fluorescence bursts at potentials negative of the equilibrium potential and many fluorescence bursts at more
positive potentials. Further SMSEC work was done with cresyl violet molecules immobilized on a transparent clay film.\textsuperscript{54}

Work by Zhao et al. \textit{(vide supra, Section 1.3.2)} exploited nanophotonic ZMW architectures to investigate homogeneous electron transfer dynamics of sarcosine oxidase, both with its nominal substrate, N-methylglycine (sarcosine), as well as, a non-canonical substrate, L-proline.\textsuperscript{32} These experiments were extended to the use of the optical cladding layer of the ZMW as the working electrode in a spectroelectrochemical cell in studies of surface functionalized flavin adenine dinucleotide (FAD).\textsuperscript{33}

1.5 Flavin Molecules

1.5.1 Role in Disease and Homeostasis

Flavin compounds are an important class of enzymatic cofactors and have been studied extensively, particularly among the oxidoreductases.\textsuperscript{55-56} Flavin-containing compounds have been implicated in human diseases including trimethylaminuria.\textsuperscript{57} They contribute to the photorepair of DNA,\textsuperscript{58} regulation of biological clocks,\textsuperscript{59} and cellular processes,\textsuperscript{60-62} among a host of other biological functions.

1.5.2 Key Attributes for Study

Flavin molecules contain the isoalloxazine chromophore, which is capable of undergoing $2e^-\text{-}2H^+$ oxidation-reduction reactions.\textsuperscript{55} The flavoprotein-derived compounds, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are derivatives of the isoalloxazine-containing riboflavin. In the oxidized state, FMN, the
chromophore is fluorescent with a moderate quantum efficiency. The reduced state, $\text{FMNH}_2$, has a significantly lower quantum efficiency and is considered a dark-state. Despite a weak dependence of the quantum efficiency on pH, the electrochemical behavior is highly dependent on pH.\textsuperscript{63-64} The presence of the adenine moiety and the availability of a hinge-motion mediated intramolecular charge-transfer process leads to a lower quantum efficiency of FAD compared to FMN in free solution.\textsuperscript{55} These spectroscopic and electrochemical properties make flavins, particularly FMN, well-suited for monitoring single-molecule fluorescence under active electrochemical potential control. Figure 1.1 shows an oxidation-reduction scheme for FMN-$\text{FMNH}_2$, with the possible electron-transfer and protonation/deprotonation reaction pathways between the oxidized state and various one-electron reduced radical semiquinone and two-electron fully reduced states.
Figure 1.1: Oxidation-reduction mechanisms of FMN in aqueous solution at pH 3-5. The proposed favored pathway is noted by solid reaction arrows; other pathways are noted by dashed reaction arrows. Relative quantum efficiencies are roughly represented with either a yellow background denoting brightly fluorescent, a yellow-gray background denoting weakly fluorescent, or a gray background denoting not fluorescent. Adapted from Tan et al.65
1.6 Objective of Work

The work here seeks to converge the topics discussed in Sections 1.2-1.5 by utilizing a fluorescence correlation spectroscopy optical system to study flavin molecules freely diffusing in zero-mode waveguides by means of single-molecule spectroelectrochemistry. The purpose of this work is to build on the progress made by Bohn and coworkers in recent years towards understanding the effects of confinement and congestion at the nanoscale on molecular transport and molecular reactivity.

1.7 Organization of the Text

The following work highlights further development in the control and observation of redox molecules freely diffusing in a confined environment. In Chapter 2, the design of a fluorescence correlation spectroscopy (FCS) optical system, its construction, and optimization for fluorescence measurements, is detailed. The focus is on the design and optimization of the excitation and detection pathways for highly sensitive measurements, both spatially and temporally.

Building on the work in Chapter 2, the focus of Chapter 3 is on hardware upgrades, software integration, and technique development necessary for the measurement of molecules inside of zero-mode waveguides (ZMWs). Attention is given to the experimental progression necessary to image a ZMW array and colocalize the optical observation volume inside a single ZMW.

Chapter 4 discusses the results attained through utilization of the upgraded instrument described in Chapter 3. Flavin mononucleotide (FMN) is studied under a
range of static and temporally-varying potential control programs that elucidate key properties of its behavior. Bulk measurements atop indium tin oxide (ITO) are compared to confined measurements in Au-clad ZMWs. The rapid response rate of ZMW-based measurements is demonstrated.

Opportunities for expansion of the technique are considered alongside challenges and limitations with suggested paths for further growth in Chapter 5. These include improvements to the optical system, changes in the experimental design, and lastly, other chemical systems. Final remarks are made concerning the outlook for integration of nanoscale features in LOC and POC devices.
CHAPTER 2:
FLUORESCENCE CORRELATION SPECTROSCOPY OPTICAL SYSTEM

2.1 Introduction

The theoretical basis and foundational implementation of fluorescence correlation spectroscopy (FCS) was detailed in Section 1.2. A confocal microscope configured for FCS is described here. Various customizations were performed to address non-idealities and increase the measurement accuracy.

2.2 Confocal Microscope Design

2.2.1 Criteria and Principles

A confocal microscope was custom built to record fluorescence intensity traces and perform fluorescence correlation spectroscopy experiments. Several design principles guided the selection of optics and construction. Due to the low signal levels inherent to single-molecule microscopy, the primary design criterion was to maximize detection of emitted fluorescence photons. A secondary design criterion was to make the system insensitive to changes in excitation and emission wavelengths over a large portion of the visible range of the electromagnetic spectrum. This made the instrument amenable to a wide range of chemical systems. The primary design principle to meet the
criterion of maximum detection efficiency was to minimize the number of optics needed to achieve each of the optical transformations in the detection pathway. This principle was also used in the excitation pathway, but primarily to keep the system simpler and reduce the number of failure modes. In order to make the system achromatic, achromatic doublet lenses were used whenever possible. Because fluorescent molecules emit over a range of wavelengths, this choice was consistent with the primary design criterion.

Other considerations in the design included vibration damping and background signal minimization. The system was built on a passively damped optical table and vibrations were damped by: (1) keeping optical components close to the table, (2) using a minimum number of opto-mechanical components to position each optic, (3) using static opto-mechanical components when possible, (4) mounting optics using no more than three nodes between the table (or optical breadboard) and its final position, (5) shortening optical path lengths, and (6) suspending all accessory equipment (e.g., computer towers, voltage supplies, cabling, etc.) on shelving above the table.

Background signals were minimized through a system of light baffling, which also provided a number of other benefits, detailed below. An additional consideration was to reuse as many components as possible from previously constructed instruments in the laboratory; these will be detailed below.
2.2.2 Optical Formulas

The probe volume can be described with a first-order approximation as a 3D Gaussian shape, where the intensity is expressed as,

\[
I(x, y, z) = I_0 \exp \left[ -\frac{2(x^2 + y^2)}{r_0^2} - \frac{2z^2}{z_0^2} \right]
\]  

in three dimensions, where \( r_0 \) is the radius at the beam waist and \( z_0 \) is the axial half-height. Figure 2.1 illustrates the approximate shape of such a probe volume.

![Diagram](image)

Figure 2.1: Schematic illustration of the diffraction-limited spot produced when a collimated beam enters and slightly overfills the back aperture of a microscope objective. The blue hourglass shape represents the illumination profile in two-dimensions. The green ellipse represents the confocal detection probe volume in two-dimensions with radius \( r_0 \) and axial half-height \( z_0 \). The blue-green gradient below and above the objective represents the epi-illumination configuration.
The minimum $r_0$ and $z_0$ values can be initially estimated as the limits of the radial and axial resolution, calculated in Equations 2.2 and 2.3, respectively,

$$r_{\min} = r_{\text{Airy}} = \frac{0.61\lambda}{NA_{\text{obj}}} = \frac{0.61(488\text{nm})}{1.3} = 229 \text{ nm}$$  \hspace{1cm} (2.2)

$$z_{\min} = \frac{2\lambda\eta}{(NA_{\text{obj}})^2} = \frac{2(488\text{nm})(1.52)}{(1.3)^2} = 877 \text{ nm}$$  \hspace{1cm} (2.3)

where $\lambda$ is the wavelength of the excitation light, $NA_{\text{obj}}$ is the numerical aperture of the objective, and $\eta$ is the refractive index of the object medium. Integrating Equation 2.1 results in the volume of the point spread function (PSF) of the probe volume,

$$V_{\text{PSF}} = \int \left[ \frac{I(x, y, z)}{I_0} \right] dx dy dz = r_0^2 z_0 \left( \frac{\pi}{2} \right)^{3/2} = 0.0905 \text{ fL}$$  \hspace{1cm} (2.4)

and using the $r_{\min}$ and $z_{\min}$ values results in an approximately 0.1 fL, as used above. As can be seen through Equations 2.2-2.4, the probe volume is determined primarily by the characteristics of the objective used and the wavelength.

2.3 Confocal Microscope Construction

2.3.1 Principles and Description of the First Instrument

A well-functioning confocal microscope requires a precise combination of optics, well-aligned to one another to excite fluorescent molecules and direct that fluorescence to a detector. Each additional optic and optical transformation introduces an additional failure mode into the system and reduces the number of transmitted photons. Thus, a
guiding construction principle was to start with the simplest design, and to that, incrementally add complexity to increase capabilities. One of the simplest confocal microscope setups possible with commercially available optics is described as follows:

1. A laser outputs a collimated monochromatic excitation beam,
2. A two-lens beam expander enlarges the collimated beam diameter to just overfill the diameter of the back aperture of the objective,
3. A dichroic filter reflects the excitation beam into the back aperture of the objective,
4. The objective focuses the excitation beam to a diffraction-limited spot in the sample plane,
5. The objective collects fluorescence emitted by the sample and directs it in a collimated beam back to the dichroic filter,
6. The dichroic filter transmits longer fluorescence wavelengths and reflects the shorter excitation wavelength back into the excitation pathway,
7. A mirror directs the fluorescence beam into the detection pathway,
8. An emission filter transmits selected fluorescence wavelengths and rejects scattered photons at the excitation wavelength,
9. A tube lens focuses the fluorescence beam onto a confocal pinhole,
10. The confocal pinhole rejects out-of-focus light, effectively reducing the size of the optical probe volume,
11. A second tube lens collects the fluorescence transmitted by the pinhole and focuses it onto the active area of a detector,
12. The signal from the detector is recorded by an electronic acquisition device.

This basic confocal microscope, represented in Figure 2.2, was the foundation upon which upgrades and improvements were made. Its working order was established
by measuring the response of fluorescence intensity, $I_i$, to changes in excitation intensity.

Figure 2.2: Schematic of the first confocal FCS instrument. It was constructed with a minimum number of optical and electronic components, and formed the design from which all upgraded instruments described here were built.

2.3.2 Expansion of Capabilities

2.3.2.1 Neutral Density Filters

As is always the case with fluorescence-based studies, photobleaching is a major concern.\textsuperscript{68-69} Even with losses due to absorbance and reflection in the excitation pathway, the total power reaching the sample plane has been observed experimentally to be greater than 20% of the power output by the laser. An output laser power of 1 mW translates to an irradiance as high as $4.9 \times 10^3 \mu W/\mu m^2$ at the diffraction-limited beam waist in the sample plane, or simply 200 $\mu W/probe$ area. Many groups report
using excitation powers of ca. 10-100 μW in the sample plane.\textsuperscript{70-71} Neutral density filters directly after the laser were used to uniformly attenuate the laser power; here, effectively reducing the laser’s power output range to 200 μW to 2 mW. Doing so also makes alignment of the excitation pathway safer by reducing the maximum intensity, and easier, by bringing the intensity closer to the dynamic range of the eye.

2.3.2.2 Spatial Filter

An assumption of correlation fitting models is that the intensity distribution of the optical probe volume is a symmetric 2D Gaussian distribution in its horizontal and vertical planes, giving the volume a 3D Gaussian distribution.\textsuperscript{12} The optical input needed to achieve this is a fundamental transverse electromagnetic (TEM) mode TEM\textsubscript{00} Gaussian input into the objective.\textsuperscript{72} Though the output of the laser is specified to be TEM\textsubscript{00}, the sensitivity of FCS analysis to the transverse mode structure warrants the use of a spatial filter to ensure a TEM\textsubscript{00} input for the objective. The spatial filter also served as an initial beam expander, reducing the magnification that needed to be performed by the dedicated beam expander that followed.

2.3.2.3 Polarizers

The polarization state also has a significant impact on the shape of the probe volume. Woehl and coworkers extensively investigated the effects of illumination polarization, stratified media, and deviations from design and ideal values on the shape of the illumination point spread function (PSF) of a confocal microscope.\textsuperscript{73} Their
experimental measurements of the PSF in three dimensions allowed them to verify the
calculations of a theoretical model that takes into account all of the variables they
investigated.\textsuperscript{73-74} Notably, they observed a dissymmetry along the axial dimension that is
often overlooked in the literature on confocal detection.\textsuperscript{73} Their simulations suggest
that slightly underfilling the objective is more favorable for FCS because it produces a
smoother PSF than the more tightly focused beam with an overfilled objective.\textsuperscript{74} PSF
Lab, a program developed by Woehl et al., was used to simulate the effects of different
excitation polarizations on the illumination point spread function of this instrument.
Table 2.1 summarizes the variables used by the simulation.
TABLE 2.1

VARIABLE STATES USED IN NUMERICAL SIMULATION OF ILLUMINATION POINT SPREAD FUNCTIONS OF THE INSTRUMENT

<table>
<thead>
<tr>
<th>Variable state</th>
<th>Design</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Objective NA</td>
<td>-</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>-</td>
<td>488</td>
<td>488</td>
</tr>
<tr>
<td>Filling factor, objective aperture radius/ beam waist radius</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Distance between objective and coverslip</td>
<td>160</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>Coverslip thickness (μm)</td>
<td>170</td>
<td>170</td>
<td>170</td>
</tr>
<tr>
<td>Refractive index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immersion oil</td>
<td>1.518</td>
<td>1.518</td>
<td>1.518</td>
</tr>
<tr>
<td>Coverslip glass</td>
<td>1.515</td>
<td>1.5255</td>
<td>1.5255</td>
</tr>
<tr>
<td>Aqueous sample</td>
<td>1.33</td>
<td>1.33</td>
<td>1.33</td>
</tr>
<tr>
<td>Polarization</td>
<td>-</td>
<td>Linear</td>
<td>Circular</td>
</tr>
<tr>
<td>Depth into sample (μm)</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2.3 shows the results of the numerical simulations done in PSF Lab with the variables in Table 2.1. It is immediately apparent that circular polarization results in a completely symmetric Gaussian PSF cross-section at the beam waist, compared to the elliptical PSF cross-section produced by linearly polarized excitation light. The polarization does not change the axial shape of the beam, but a stark dissymmetry is apparent about the beam waist in both models. Despite the target focus being set at the glass-liquid interface, corresponding to a depth of 0 μm, the focus occurs just beyond 1.5 μm into the liquid sample. Additional simulations showed that the small difference
between the optimal refractive index value of 1.515 for the coverslip glass and the actual value of 1.5255 is solely responsible for the discrepancy, illustrating the importance of attention to detail in experimental setup. The \( r_0 \) value of 194 nm is 15.3% smaller than the theoretical \( r_0 \) value calculated by Equation 2.2. The \( z_0 \) values of 366 and 959 nm average to 663 nm, 24.4% smaller than the theoretical \( z_0 \) value calculated by Equation 2.3. These disagreements are minor, especially considering that these values serve only as minimum boundaries for fitting in autocorrelation and cross-correlation models where the values are determined empirically. In response to these simulations, the laser beam was filtered through a vertically-aligned linear polarizer (5511, Newport Corporation, Irvine, CA) and passed through a zero-order quarter-wave plate (05RP04-12, Newport Corporation, Irvine, CA) with its fast-axis at 45°, transforming the polarization of the beam from vertical linear to circular. Jones calculus was performed to confirm the angular placement of these optics. \(^{75}\)
Figure 2.3: Heat maps showing illumination intensities: (a) x-y plane of the illumination PSF model using linear polarization, (b) x-y plane using circular polarization, (c) x-z plane using linear polarization, and (d) x-z plane using circular polarization. All plots are indexed to the same intensity peak and the 1/e^2 radii and half-heights are denoted.
2.3.2.4 Raised Breadboard

Though vibration damping was critical throughout the instrument, clearly, the area most sensitive to the effects of vibration, air currents, and other environmental non-idealities was the vertically-aligned sample column. Because all devices for testing must incorporate a 170 μm thick coverslip, the sample cannot be as securely locked into place as the optics and all other system components were, thus providing a source of drift and susceptibility to vibrations. Additionally, the sample, sample stage, objective, and objective stage all had to be suspended at least 30 cm above the table to provide room for the dichroic filter and mirror beneath them. Because keeping all optical components close to the table was important for vibration mitigation, an optical breadboard was designed to address these concerns. It was custom manufactured (Custom RG Series Breadboard, Newport Corporation, Irvine, CA) and incorporated into the instrument. It was firmly secured to the vibration-isolation table by four thick posts and contains a trussed honeycomb core similar to the table itself. The objective stage and sample stage were both affixed to this breadboard to lessen the effects of vibrations. The design schematic for the breadboard is seen in Figure 2.4.
2.3.2.5 Emission Filters

Finally, two long pass emission filters were added to the microscope. The advent of a new commercial coating process delivered near lossless emission filters in the allowed wavelength range, greatly reducing fluorescence intensity losses, while providing additional rejection of the excitation wavelength. Figure 2.5 shows representative transmission spectra of the filters used in the microscope.
2.3.2.6 Baffling

Apart from the microscope, but important to its operation, is a custom light baffling assembly made of foam board with a black matte paper exterior. It primarily serves to reduce the observed background signal by increasing the number of reflections necessary for off-axis photons to reach the detector. The outer baffling assembly surrounds the instrument and enhances safety by enclosing the laser radiation, preserves alignment by providing a physical barrier that prevents accidental bumping of optics and opto-mechanics, and reduces dust with its roof. An easily removable panel in the exterior baffling provides access to the sample column, as seen in Figure 2.6. A baffling wall separates the excitation and detection sides of the system, just to the left of the sample column, between the second and third emission filters.
This wall prevents off-axis laser radiation from entering the optical pathway on the detection side. Interior baffling around the detection pathway, seen in Figure 2.7, provides additional discrimination against stray photons with panels between most of the optics in the detection pathway. Though experiments are run with the dark curtain closed and the room lights off, the increase in background signal is negligible with the room lights on when all of the baffling is in place. The baffling enables use of computer monitors during data acquisition with no increase in the background signal. Schematics showing the placement of exterior and interior baffling panels relative to the vibration table and to one another are shown in Appendix A.

Figure 2.6: Exterior baffling encloses the system and allows for quick and easy access to the sample column.
2.3.3 Description of the Microscope

2.3.3.1 Excitation Pathway

The microscope was built atop a passive damped optical table (RS2000-510-12, Newport Corporation, Irvine, CA) supported by four pneumatic legs (SL Series LabLegs, Newport Corporation, Irvine, CA). A 488 nm solid-state continuous laser (Sapphire 488-20, Coherent, Inc., Santa Clara, CA) delivered a TEM\textsubscript{00} beam linearly polarized along the vertical axis with a $1/e^2$ diameter of 0.70 ± 0.05 mm. Adjustable output power ranged from 1-20 mW. A pair of neutral density filters (FSR-OD30, FSR-OD70, Newport Corporation, Irvine, CA) were stacked and mounted on a kinematic flip base (PS-KF, Newport Corporation, Irvine, CA), allowing them to be reliably moved in and out of the beam pathway. Their optical densities of 0.3 and 0.7 translated to transmittances of 50.12% and 19.95%, respectively, or 10.00% when used together. The beam was incident on a dielectric mirror (5101, Newport Corporation, Irvine, CA) oriented at 45° to...
direct the radiation into the back aperture of a 20x, 0.40 NA, 9.0 mm effective focal length air objective (M-20X, Newport Corporation, Irvine, CA) as part of a three-axis spatial filter (900, Newport Corporation, Irvine, CA). A pinhole (900PH-15, Newport Corporation, Irvine, CA) placed at the focal point of the beam allowed only the TEM$_{00}$ portion of the beam to pass, excluding higher-order transverse modes. A 38.1 mm effective focal length, 430-700 nm anti-reflection coated achromatic doublet lens (PAC025AR.14, Newport Corporation, Irvine, CA) collected and collimated the diverging output beam. The collimated beam then passed through a vertically-aligned sheet polarizer (5511, Newport Corporation, Irvine, CA) to clean up the linear polarization. The polarization was transformed to circularly polarized by a zero-order quarter-wave plate (05RP04-12, Newport Corporation, Irvine, CA). The beam was then directed by a second 45° dielectric mirror (5101, Newport Corporation, Irvine, CA) to a 76.2 mm effective focal length, 430-700 nm anti-reflection coated achromatic doublet lens (PAC046AR.14, Newport Corporation, Irvine, CA) as the first lens in a Keplerian beam expander. The second beam expander lens, a 250 mm effective focal length, 430-700 nm anti-reflection coated plano-convex lens (KPX202AR.14, Newport Corporation, Irvine, CA), collected and collimated the beam, resulting in an approximately 3.28x expansion of the beam. The expanded beam was directed by a 45° dichroic filter (Z488RDC, Chroma Technology Corporation, Bellows Falls, VT) upward along the z-axis into the back aperture of a 40x, 1.30 NA, oil immersion objective (440255-9901-000, Carl Zeiss, Inc., Jena, Germany). The 1/e$^2$ diameter was approximately 9.72 mm exiting the beam expander, slightly smaller than the 10 mm back aperture of the objective. The objective
then focused the beam to a diffraction-limited spot at the sample plane. A high-precision 170 ± 5 mm thick glass coverslip (0107242, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) served as the final optic in the excitation pathway. Figure 2.8 shows a photograph of the excitation side. A photograph of the mechanical slide holder (XYFM1, Thor Labs, Newton, NJ) mounted to the sample stage is shown in Figure 2.9.

Figure 2.8: The instrument’s excitation side from the laser on the right to the sample column on the left.
2.3.3.2 Detection Pathway

Fluorescence from the sample was collected through the glass coverslip by the objective in an epi-illumination configuration. The emitted photons passed through the dichroic filter and were directed by a third 45° dielectric mirror (5101, Newport Corporation, Irvine, CA) to propagate parallel to the table. Residual scattered laser photons were eliminated by a long pass emission filter (ET500lp, Chroma Technology Corporation, Bellows Falls, VT), a band pass emission filter (ET525/50m, Chroma Technology Corporation, Bellows Falls, VT), and a second long pass emission filter (ET500lp, Chroma Technology Corporation, Bellows Falls, VT). The collected fluorescence was focused by a 76.2 mm effective focal length, 430-700 nm anti-reflection coated achromatic doublet lens (PAC046AR.14, Newport Corporation, Irvine, CA) onto a pinhole (910PH-30, Newport Corporation, Irvine, CA). The confocal pinhole
served to eliminate any out-of-focus radiation, drastically reducing the background contribution to the detected signal. A 50.8 mm effective focal length, 430-700 nm anti-reflection coated achromatic doublet lens (PAC050AR.14, Newport Corporation, Irvine, CA) was placed at twice its effective focal length and collected diverging photons and focused them down to the 175 μm diameter active area of an avalanche photodiode (SPCM-AQR-16, PerkinElmer, Inc., Waltham, MA). The detected photons were recorded as integer signal pulses by a multi-channel scalar card (MCS-pci, Ortec, Oak Ridge, TN). Figure 2.10 shows a photograph of the detection side of the instrument. A schematic diagram of the entire instrument is found in Figure 2.11.

Figure 2.10: The instrument’s detection side from the sample column on the right to the APD on the left.
2.4 Alignment Techniques

2.4.1 Principles

Each optic in the system has six degrees of freedom, though the number of controllable degrees of freedom depends on the opto-mechanical component in which they are mounted. Due to the sheer complexity of optimizing the alignment of a system with over one-hundred degrees, a general alignment principle is to fully align each optic before moving onto the next optic.

2.4.1.1 Theoretical Placement and Experimental Tuning

Each optic has a theoretical placement in relation to the optics immediately preceding and following it. When the beam exiting an optic is collimated, the distance
between that optic and the one that follows can vary without effect. The distance between optics is important when the beam’s diameter varies. This is the case, for instance, with the two lenses composing the beam expanding on the excitation side. The lenses have focal distances of 76.2 and 250 mm, respectively. Accordingly, they are initially placed 326.2 mm apart from each other, f-number matching them. This condition produces a roughly collimated beam that is more finely tuned by varying the distance and evaluating the collimation with a shearing interferometer. Each optic is placed and adjusted primarily with the methods described below in Sections 2.4.1.2 and 2.4.1.3.

2.4.1.2 Aperture Aiming

Once an optic is roughly placed, it is translated and rotated to direct the laser onto the same point in the center of an adjustable aperture, secured at a constant height. This is observed at a point directly after the optic and at a point far from the optic on the same optical axis. The laser light is first directed to the far aperture by changing the angle and z-position of the optic. The laser light is then directed to the near aperture by changing the x-position and y-position of the optic. This is repeated iteratively until the beam intersects the same position on the aperture at both positions. The constant height of the aperture is set on the excitation side by the laser and on the detection side by the APD. The right-hand rule is used to determine an axis of rotation. On the detection side, a bright beam of fluorescence, resulting from an illuminated
highly fluorescent solution, is used in place of the laser to align the detection side by eye.

2.4.1.3 Intensity Measurements

The intensity of the beam is measured at the exit of each optic in the excitation pathway using a power meter (PM100D, S130C, Thor Labs, Newton, NJ). The intensity after each optic provides an indirect measure of the quality of alignment. Mirrors and lenses are expected to transmit the incident beam with only a small intensity loss. Pinholes, filters, and polarizers are all expected to exhibit greater losses because they are designed to selectively transmit a portion of the incident beam. For these optics, the recorded intensity is maximized through fine adjustments of the opto-mechanics. Intensity losses at each optic can be compared to a record of losses from previous alignment iterations to ensure that the new loss values are reasonable. Measurement of the intensity serves as an important factor in formation of the optical probe volume. A highly fluorescent solution is used to observe the shape of the beam exiting the objective. Though the diffraction-limited spot is too small to resolve with the eye, the beam diverges and the optics preceding the objective can be adjusted to ensure that the diverging cone is symmetrical in $360^\circ$. This is done iteratively with maximization of the recorded intensity. The process is finished when no improvement is observed in either the symmetry or intensity.

In the detection pathway, the intensity is measured at the active area of the APD directly by the APD itself. A photostable fluorophore, such as Alexa Fluor® 488, is used
at the lowest concentration possible to produce a relatively constant signal when excited. The optics in the detection pathway are iteratively adjusted along various degrees-of-freedom until the fluorescence signal is maximized.

2.5 Optimization and Calibration Data

Numerous chemical systems, all with strong absorption along the 488 nm laser line, were used throughout the alignment, optimization, and calibration of the instrument. These include low molecular weight dyes (e.g., fluorescein, fluorescein isothiocyanate (FITC), Alexa Fluor® 488), medium to high molecular weight macromolecules (e.g., FITC-dextran, fibrinogen-Alexa Fluor® 488 conjugate), and fluorescently-labeled nanospheres (i.e, carboxylate-modified FluoSpheres®). Due to the negative net surface charge on borosilicate glass and polydimethylsiloxane (PDMS) when used with aqueous solutions, fluorophores and fluorophore-tagged macromolecules with negative net surface charges were chosen to deter surface adhesion. Reported values of the diffusion coefficient or the hydrodynamic radius of each compound can be used to hold $D$ constant in Equation 1.6 during fitting of the correlation curve, enabling estimation of the probe volume parameters. The value of $D$ can be estimated from the hydrodynamic radius using Stokes-Einstein Equation,

$$D = \frac{kT}{6\pi \eta r_h}$$  \hspace{1cm} (2.5)

where $k$ is Boltzmann’s constant, $T$ is the absolute temperature, $\eta$ is the viscosity of the media, and $r_h$ is the hydrodynamic radius of the molecule. The underlying assumptions
of the equation hold best for small dyes and globular macromolecules because their physical and their hydrodynamic radii are similar. The equation can be used for aspherical molecules like dextran if the \( r_h \) value used was determined under similar experimental conditions.

2.5.1 Raw Data

Two data sets acquired with the instrumental configuration in Figure 2.11 are discussed here. The first chemical system is of freely diffusing 40 nm diameter carboxylate-modified FluoSpheres® (F-8795, Life Technologies, Carlsbad, CA) at a concentration of \( 10^{-3} \) spheres/fL in 5 mM borate buffer at pH 9.0. Five 60 s acquisitions were concatenated to produce one 300 s acquisition. As is shown in Figure 2.12, the FluoSpheres® emit brightly, producing a high signal-to-background ratio of more than 10 for the most intense bursts of fluorescence. Treating the physical radius of 20 nm as the hydrodynamic radius, Equation 2.5 estimates \( D \approx 1.07 \times 10^{-7} \text{ cm}^2/\text{s} \) at 20 °C. Using Equation 1.5, and treating the \( r_{\text{min}} \) value of 229 nm calculated in Equation 2.2 as \( r_0 \), this \( D \) value predicts a \( \tau_D \) value of \( \approx 0.82 \text{ ms} \). The subsets of the data, in particular Figure 2.12(c), show what is likely numerous recrossings of an aggregate of FluoSpheres® through the probe volume. The shortest residence time centered at about 164.36 s, lasts a \( \approx 10 \text{ ms} \), an order of magnitude larger than the predicted \( \tau_D \) value. The fit to the autocorrelation of this data leads to a similar conclusion in Section 2.5.3.
Figure 2.12: Fluorescence intensity traces of $10^{-3}$ spheres/fL 40 nm diameter FluoSpheres® in 5 mM borate buffer at pH 9.0; (a) full range of data, (b) 10 s subset of data in (a), and (c) 500 ms subset of data in (a). Data was recorded in 1 ms time bins. A 1,001-point boxcar smoothing routine was applied to the raw data to produce the overlaid black trace in (a).

The second chemical system is of 100 pM 4,000 g/mol FITC-dextran (FD4, Sigma-Aldrich Corporation, St. Louis, MO) in 25 mM borate buffer at pH 9.0. Five 60 s acquisitions were concatenated to produce one 300 s acquisition. At this concentration and in an optical probe volume calculated by Equation 2.4, the average molecular occupancy, $\langle N \rangle$, is predicted to be $5.4 \times 10^{-3}$ molecules. A fluorescence intensity trace with subsets of data is seen in Figure 2.13. Using the relationship derived by Shao and Baltus, $D$ is estimated to be $1.36 \times 10^{-6}$ cm$^2$/s at 20 °C. Using Equation 1.5, and treating
the $r_{\text{min}}$ value of 229 nm calculated in Equation 2.2 as $r_0$, this $D$ value predicts a $\tau_D$ value of $\approx 64$ $\mu$s. The subsets of the data reveal a signal-to-background ratio similar to that for the FluoSphere® data, but with significantly lower absolute signal intensity. The burst of fluorescence in Figure 2.13(c), has a full width at half maximum (FWHM) of $\approx 2$ ms. The fit to the autocorrelation of this data, in Section 2.5.3, suggests that this is an overestimate of $\tau_D$.

![Figure 2.13](image.png)

Figure 2.13: Fluorescence intensity traces of 100 pM 4,000 g/mol FITC-dextran in 25 mM borate buffer at pH 9.0; (a) full range of data, (b) 2 s subset of data in (a), and (c) 100 ms subset of data in (a). Data was recorded in 1 ms time bins. A 101-point boxcar smoothing routine was applied to the raw data to produce the overlaid black trace in (a).
2.5.2 Histograms and Poisson Fits

Evaluation of the photon counting histograms of fluorescence intensity traces serves as a statistical indication of meaningful bursts of fluorescence. Purely random noise, whether from a stable background signal, dark counts, or shot noise will have a Poissonian distribution.\textsuperscript{79} Fitting Equation 1.7 to a histogram of a fluorescence intensity trace can reveal a super-Poissonian distribution, i.e. one with a significant number of events lying outside the distribution. Such a distribution indicates a contribution to the signal that is not due to the background. In general, the more counts that lie above and to the right of the Poisson fit, the more likely the data set contains events resulting from single-molecule fluorescence.

Figure 2.14 shows the histogram of Figure 2.12 and the resulting Poisson distribution fit. A super-Poissonian distribution is apparent, as is expected considering that the fluorescence intensity trace in Figure 2.12 clearly shows spikes well above the background level.
Figure 2.14: Photon counting histogram of FluoSphere® data in Figure 2.12 and the resulting Poisson distribution fit. The expectation value, $\lambda$, determined the Poisson fit, is $1.30 \pm 0.04$ counts/ms.

Figure 2.15 shows the histogram of Figure 2.13 and the resulting Poisson distribution fit. A super-Poissonian distribution is apparent, but not as clear as it is in Figure 2.14. Noting that the histograms are displayed with a logarithmic scale, only a small fraction of the 300,000 time bins are super-Poissonian in Figure 2.15. Two similar looking intensity traces may yield drastically different photon counting histograms, one with and one without a super-Poissonian distribution.
Figure 2.15: Photon counting histogram of FITC-dextran data in Figure 2.13 and the resulting Poisson distribution fit. The expectation value, $\lambda$, determined the Poisson fit, is $0.22 \pm 0.02$ counts/ms.

2.5.3 Autocorrelation Data

As discussed in Section 1.2, the primary value that FCS adds to the suite of fluorescence-based techniques is the information that can be derived from correlation of fluorescence data. The data presented in Section 2.5.1 was autocorrelated with a software procedure using IGOR Pro (IGOR Pro 6, WaveMetrics, Inc., Lake Oswego, OR). Figure 2.16 shows the autocorrelation curve of the FluoSphere® data in Figure 2.12. The fit using the 3D-diffusion model in Equation 1.6 appears to describe the curve well, except below 10 ms. The $D$ value of $1.59 \times 10^{-9}$ cm$^2$/s from the fit is 1.5% of the expected $D \approx 1.07 \times 10^{-7}$ cm$^2$/s, supporting the FluoSphere® aggregation hypothesis suggested in Section 2.5.1. The $r_0$ and $z_0$ values are reasonable, both being well within an order of magnitude of the values calculated in Equations 2.2 and 2.3.
Figure 2.16: Autocorrelation curve of the FluoSphere® data in Figure 2.12 with the corresponding 3D-diffusion model fit obtained with Equation 1.6.

Figure 2.17 shows the autocorrelation curve of the FITC-dextran data in Figure 2.13. The fit using the 3D-diffusion model in Equation 1.6 was constrained with a value of 229 nm for $r_0$; it appears to describe the entire curve well, but produces a value of $z_0$ too large by two orders of magnitude. The $D$ value of $1.29 \times 10^{-6}$ cm$^2$/s from the fit is 94.9% of the expected $D \approx 1.36 \times 10^{-6}$ cm$^2$/s.
Figure 2.17: Autocorrelation curve of the FITC-dextran data in Figure 2.13 with the corresponding 3D-diffusion model fit obtained with Equation 1.6.

Clearly, both data sets present non-idealities, particularly in relation to calibration of the instrument for reliable correlation measurements. Figure 2.18 shows the ability of the FCS instrument to make valid relative comparisons even when it is out of calibration. The autocorrelation curves of three different molecular weights—4,000, 10,000, and 40,000 g/mol (FD4, FD10S, and FD40S, Sigma-Aldrich Corporation, St. Louis, MO)—of FITC-dextran at a concentration of either 100 pM or 1 nM in 25 mM borate buffer at pH 9.0 are shown. The curve for 4,000 g/mol FITC-dextran is the same as in Figure 2.17. As is expected, the autocorrelation shifts to longer diffusion timescales as the molecular weight increases, corresponding to an increase in the hydrodynamic radius, and thus, a smaller diffusion coefficient.
Figure 2.18: Autocorrelation curves of 4,000, 10,000, and 40,000 g/mol FITC-dextran with their corresponding 3D-diffusion model fits obtained with Equation 1.6.

2.6 Concluding Remarks

The design, construction, and alignment of the FCS optical system described here were performed in a systematic way. Additional capabilities were added iteratively in order to isolate their resulting effects on instrument operation. A basic alignment procedure was formalized with customizations appropriate to each optic and desired optical transformation. Proper alignment was evaluated in the most direct way possible. Measurements of fluorescence from single-molecule concentrations of various chemical systems were used to evaluate the performance of the instrument and determine which areas needed improvement. System upgrades and measures to address performance inadequacies are addressed in Chapter 3.
CHAPTER 3:

FLUORESCENCE CORRELATION SPECTROSCOPY OPTICAL SYSTEM UPGRADES

3.1 Introduction

The instrument described in Chapter 2 provided a foundation to which additional capabilities could be added, improvements made, and techniques refined for different applications. As was the approach when building the instrument, components and capabilities were added incrementally, so that non-idealities could be systematically addressed.

3.2 Addition of Imaging Pathway

Experiments using glass or polydimethylsiloxane (PDMS) wells on a coverslip were readily set up with two adjacent wells, one containing the low concentration target sample and the other containing a more concentrated fluorophore solution. Placement of the well, at least 1 mm in diameter, over the exit aperture of the objective, was done by eye and the quality of the focus was determined through the signal level of the high fluorescence intensity sample. Investigation of microchannels, nanochannels, and nanopores requires imaging capabilities to colocalize the optical probe volume and the region of interest, particularly when the diameter of the feature is smaller than the diameter of the probe volume, as is the case with nanopores. In experimental
configurations where it was not possible to introduce a more concentrated sample, either in an adjacent feature or by flowing a concentrated sample through the region of interest, a coarse focus was achieved through an independent imaging pathway. Figure 3.1 shows a schematic of the confocal microscope with the imaging pathway engaged. A slide-in mirror (5101, Newport Corporation, Irvine, CA) intercepts the detection pathway between the first and second emission filters and sends the beam through a tube lens (PAC040AR.14, Newport Corporation, Irvine, CA) and into the camera lens (105mm f/2.8D AF Micro-NIKKOR, Nikon Corporation, Tokyo, Japan) of a video-rate high-resolution CCD (JE7462DC, Javelin). The image is displayed on a CRT monitor (TR-930B, Panasonic, Kadoma, Japan). Depending on the illumination source, the first emission filter can be flipped in or out of the beam’s pathway. The imaging pathway was aligned using 50 μm x 50 μm cross-section microchannels fabricated in PDMS and bonded to a glass coverslip as the target features. These were illuminated by a white light source in a transillumination configuration, analogous to bright-field illumination in an inverted microscope.
3.3 Addition of Transillumination Pathway

The addition of the imaging pathway expanded the variety of samples and features that could be investigated. Arrays of nanopores, configured as zero-mode waveguide (ZMW) arrays, were the primary target structure of interest. Due to their ability to strongly attenuate wavelengths above a critical wavelength (vide supra, Section 1.3.1), bright-field illumination proved challenging. White light sources produce a divergent beam, so in order to obtain high intensities in the sample plane, the source exit aperture must be placed close to the sample. Furthermore, the light source had to be removed when changing samples, making realignment difficult. Because the coupling of light through the nanopore features exhibits a large angular variation, there were dramatic differences in the intensity and clarity of the images produced. Additionally, a
A long-pass filter was required to select wavelengths to the red of the absorption of the fluorophore to prevent photobleaching. To bypass the latter issue, the white light source was replaced with a 10 mW 632.8 nm helium-neon laser (25-LHP-991, Melles Griot, Carlsbad, CA). This wavelength is to the red of the absorption spectra of all fluorescent molecules investigated and is transmitted by a dichroic filter. The light source was precisely positioned over the sample by a fiber optic assembly. The HeNe laser’s output was directed into a 620-640 nm optimized gradient-index (GRIN) fiber collimator connected to a single-mode optical fiber (50-630-FC, Thor Labs, Newton, NJ). At the opposite end of the optical fiber, an aspheric lens (F260FC-B, Thor Labs, Newton, NJ) collimated the exit beam to a diameter of 2.8 mm. Careful alignment of the fiber optic assembly achieved a 3 mW output from the terminal collimator. Two OD 2.0 neutral density filters (FSR-OD200, Newport Corporation, Irvine, CA) could be independently inserted into the beam pathway, one between the laser and the fiber optic assembly, and one between the fiber optic assembly and the sample, attenuating the laser power reaching the sample to ca. 30 μW with one in place or ca. 300 nW with both in place. The terminal collimator was aligned using a ZMW array. It was placed several inches above the sample, where it could remain, even during sample changes, and was adjusted until pores across the whole array were uniformly illuminated. The HeNe laser transillumination setup is depicted in Figure 3.2.
Figure 3.2: Schematic of the expanded FCS instrument with the imaging pathway engaged by the slide-in mirror and transillumination provided by a 632.8 nm HeNe laser.

3.4 Incorporation of Piezoelectric-Driven Scanning

3.4.1 Piezoelectric System Assemblies

In the first version of the instrument, the sample stage rested on top of a piezoelectric xy flexure stage (P-517.2CL, Physik Instrumente (PI) GmbH & Co. KG, Karlsruhe, Germany), and the objective, upon a piezoelectric flexure objective scanner (P-721.LLQ, Physik Instrumente (PI) GmbH & Co. KG, Karlsruhe, Germany). In order to miniaturize device geometries to have at least one sub-micron dimension, a piezoelectric stage and scanner were incorporated to position the confocal volume in a region of interest. The two piezoelectric systems were designed in a modular fashion to allow maximum flexibility for different target applications. As a result, the manufacturer provided product manuals for components individually, but no manual for the
assembled system. Figure 3.3 shows a flowchart, developed from the product manuals, diagramming how each component is connected to and communicates with other components in the system, as well as, with external hardware and software (vide infra). Each system can be independently run in either open-loop or closed-loop configuration. Though open-loop operation provides approximately twice the spatial resolution, each system was used in its closed-loop configuration to achieve superior positional stability through use of the built-in feedback sensors, while still providing sub-nm resolution. A position control module (E-509.C2A, Physik Instrumente (PI) GmbH & Co. KG, Karlsruhe, Germany) and an amplifier module (E-503.00, Physik Instrumente (PI) GmbH & Co. KG, Karlsruhe, Germany) work in unison to set the xy stage to user-defined target positions. Alternatively, a combined position control and amplifier module (E-621.LR, Physik Instrumente (PI) GmbH & Co. KG, Karlsruhe, Germany) sets the objective scanner to the user-defined target position.
Figure 3.3: Flowchart of the two piezoelectric systems used on the instrument, with the xy stage in the left column and the objective scanner in the right column, both interfaced through the same DAQ hardware and MATLAB program. The system is represented in its closed-loop configuration where a feedback loop adjusts the voltage to maintain a target position; italicized labels identify the interactions between components. Component names are bolded and Physik Instrumente part numbers are denoted with “E” for control and amplifier components, “P” for piezoelectric and sensor components, “PZ” for product manuals, and “SM” for software manuals.
3.4.2 Computer-Controlled Piezoelectric Scanning

Since the objective scanning system’s combined controller and amplifier module had the option for direct computer interfacing for digital control, it was used to run both systems via computer-controlled analog voltage signals. A custom MATLAB® (MATLAB® R2014a, The MathWorks, Inc., Natick, MA) program utilizing the built-in Data Acquisition Toolbox™ was written to interface with data acquisition (DAQ) hardware (USB-6343, National Instruments, Austin, TX). The specific DAQ hardware was chosen, because it has four analog outputs with a voltage range of ±10 V and sixteen differential analog inputs, exceeding the minimum requirements to simultaneously control three piezoelectric axes. The DAQ hardware sends and receives voltage signals between +0.0 and +10.0 V to and from the controller modules to direct each axis independently. The amplifier modules boost the control voltage by an order of magnitude to +0.0 to +100.0 V, which corresponds to the 0.0 to 100.0 μm range of each axis. Figure 3.4 illustrates the control structure that enables a user to run the piezoelectric xy stage through a MATLAB GUI, which is shown in Figure 3.5. The multi-channel scaler (MCS) acquisition device is integrated into the control structure. A stage scanning script waits for a digital trigger to be sent by the start of an acquisition; an example of the voltage trace for a serpentine scan is shown in Figure 3.6. At the end of the scan, the signal recorded from the avalanche photodiode (APD) can be mapped to the xy stage’s location on a point-by-point basis to construct a confocal scanned image. The instrument configuration used for transmission confocal scanned imaging can be seen in Figure 3.7, where the second emission filter is flipped out of the beam path to allow the laser radiation to reach the
APD. Once a focused image of the target features has been obtained by transillumination, the system can be easily switched to the epi-illumination configuration for fluorescence confocal scanned imaging, as seen in Figure 3.8. This three-step imaging process, seen in Figure 3.2, Figure 3.7, and Figure 3.8, is necessary for most geometries and experimental conditions investigated here. The maximum incident transmission power of 3 mW far exceeds the power at which fluorescent molecules can be excited in a high NA epi-illumination configuration without significant photobleaching. This provides a high signal level for the image to be coarsely focused with the transmission pathway by observing the bright-field image on the CRT, as represented in Figure 3.2. This focal plane has been observed repeatedly to be within ±50 μm of the confocal imaging plane; as stated above, the range of the objective scanner is 100 μm, so this coarse focus is done while the objective scanner is set to the middle of its range. The configuration in Figure 3.7 is used to collect transmission confocal scanned images until a sharply focused image is obtained; Figure 3.9 shows an example of this. Finally, the configuration shown in Figure 3.8 is used to obtain epi-illumination fluorescence confocal scanned images, often focused within ±10 μm of the focused transillumination confocal scanned image. From the sharply focused epi-illumination fluorescence confocal scanned image, an example of which is seen in Figure 3.10, the xy stage can be sent to the observed location of a target feature. The position and focus are then stepped in small increments to maximize the observed fluorescence signal, at which point fluorescence experiments can be carried out.
Figure 3.4: Flowchart of the software and hardware integration for control of the PI xy sample stage using MATLAB with integration of a detector and data acquisition hardware for confocal scanned imaging.
Figure 3.5: MATLAB GUI for direct position control of the PI xy sample stage. The left-hand column controls the x and y axes of the piezoelectric sample stage. Each axis can be translated from its current position by ±0.1 or ±0.01 V, corresponding to ±1 or ±0.1 μm, respectively. Additionally, the axes can be set to any voltage between +0.0 and +10.0 V. The same can be done in the second column for the z-axis, denoting the objective scanner. The “Check Current Position” will display the +0.0 to +10.0 V value of each axis in the third column. These values are also updated every time a voltage is changed in the first two columns. The right-hand column provides the variables for scanning the xy stage. The “Image Stitcher” box maps a recorded intensity trace to the corresponding stage scanning pattern; it also adjusts the image to account for the trigger pulse delay between the start of the intensity acquisition and the stage scan.
Figure 3.6: MATLAB plot showing the serpentine scanning pattern depicted for the scanning conditions set in the right panel of Figure 3.1; the scan starts at (0, 0 V) and proceeds to (10, 10 V), corresponding to a 100 x 100 μm scan.
Figure 3.7: Schematic of the expanded FCS instrument with the transillumination and confocal pathways used in conjunction with the piezoelectric sample stage to produce transillumination confocal scanned images.

Figure 3.8: Schematic of the expanded FCS instrument with the epi-illumination and confocal pathways used in conjunction with the piezoelectric sample stage to produce epi-illumination fluorescence confocal scanned images.
Figure 3.9: Transillumination confocal scanned image of a portion of a ZMW array made of approximately 100 nm top diameter nanopores filled with 1 μM Alexa Fluor® 488 in 1X, pH 7.4 PBS, which does not fluoresce at this excitation wavelength.
Figure 3.10: Epi-illumination fluorescence confocal scanned image of the same portion of a ZMW array as seen in Figure 3.9, showing the nanopores filled with 1 μM Alexa Fluor® 488 in 1X, pH 7.4 PBS, which fluoresces under these conditions. Note the 1.5x expansion in color scale relative to Figure 3.9.

3.5 Addition of Hardware Correlator

3.5.1 Effects of Greater Temporal Resolution

The arc of electronic technology development has greatly benefited FCS, especially in the amount of data which can be analyzed in one measurement. The earliest implementation of FCS by Magde and Elson used two analog signal correlators to directly record the autocorrelation of the observed fluorescent signal, integrating for up to a full day because of relatively low temporal resolution due to long integration
times, ca. 2 to 20 ms.\textsuperscript{7} Advances in electronics hardware, such as affordable multi-channel scaler (MCS) cards, and increases in computer memory capacity made it possible to record the fluorescence signal time traces directly without needing to correlate it. This allowed researchers to extend their FCS instruments beyond correlation measurements, by their nature, data reduction techniques,\textsuperscript{80} by extensively analyzing the time traces with statistical tools to draw meaning from the distributions of the photon fluxes. These techniques include photon-counting histogram (PCH) analysis,\textsuperscript{79} fluorescence intensity distribution analysis (FIDA),\textsuperscript{81} fluorescence intensity multiple distributions analysis (FIMDA),\textsuperscript{82} and fluorescence cumulant analysis (FCA).\textsuperscript{83} Researchers could take the same data and perform software correlation, which also became computationally accessible around this time.\textsuperscript{84-85} The traditional operating mode of most MCS devices is the so-called “time mode,” which records the number of photon events within a preset time bin.\textsuperscript{80} The MCS-pci cards used in the instrument described here have a minimum time bin width of 100 ns. With a maximum of $2^{16}$ consecutive channels, only 6.55 ms of data can be recorded at this maximum temporal resolution before having to save the file, clear the memory, and start a new acquisition. Though concatenation strategies have been employed to increase the number of data points over which analysis is performed,\textsuperscript{86-87} they introduce systematic errors into the data. “Photon mode,” an alternative to “time mode,” was demonstrated with custom electronics to have minimum time bins of 25 ns by means of recording the number of time intervals between photon events.\textsuperscript{80} This was particularly well-suited for single-
molecule studies where the low concentrations of fluorescent species dictate that many consecutive time intervals may have no photons arrive.

Despite the advantages and technological innovations provided by MCS cards and comparable devices, hardware correlator technology has made its own advances since Magde and Elson’s seminal work and remains the gold-standard in regards to temporal resolution. Various manufacturers provide models with ca. 1 ns temporal resolution; PicoQuant GmbH currently offers a time-correlated single photon counting (TCSPC) module with temporal resolution down to 1 ps. Though it is intuitive that better time resolution and longer acquisition times are favorable for a technique grounded in statistical analysis, Tcherniak and co-workers systematically explored the effects of these variables on experimentally-observed diffusion coefficients. Importantly, they found that the minimum lag time, $\tau_{\text{min}}$, needed to sufficiently resolve an autocorrelation curve needed to meet the condition $\tau_{\text{min}} < \frac{2}{3} \tau_D$. Their results also showed that data needed to be acquired such that $\tau_{\text{max}} > 5,000 \tau_D$. Finally, they observed that the longer acquisition time $t_{\text{avg}}$, the greater the accuracy of the measurement and that the necessary $t_{\text{avg}}$ must be determined experimentally for each set of experimental conditions. These findings supported the work of Gell et al., which stated that though software autocorrelation can produce accurate results, hardware autocorrelation can produce accurate results with orders of magnitude less $t_{\text{avg}}$. 
3.5.2 PicoQuant Hardware Correlator

A time-correlated single photon counting (TCSPC) module (PicoHarp 300, PicoQuant Photonics North America, Inc., West Springfield, MA) was evaluated, and based on data collected by the PicoHarp with the FCS in its two-channel configuration (vide infra, Section 3.6), a TCSPC system was purchased. The TimeHarp 260 PICO TCSPC and MCS board (TimeHarp 260 PICO Single, PicoQuant Photonics North America, Inc., West Springfield, MA) was chosen because of its low dead time and high sustainable count rate relative to the PicoHarp. Even though the timing resolution of the PicoHarp is 4 ps compared to the TimeHarp’s 25 ps, the TimeHarp outperforms both the MCS card’s 100 ns resolution and the approximately 500 ps resolution of the SPAD. It is also twice as good as the 50 ps timing resolution of the current market-leading SPADs (PDM Series, Micro Photon Devices, Bolzano, Italy). The TimeHarp’s PCIe interface enables faster data transfer than the PCI interface of the MCS and the USB of the PicoHarp. The PCIe can sustain time tagging up to 40 Mcps, which far exceeds the linear response region of the current Perkin-Elmer SPCM SPADs (< 6 Mcps). The dead time of the TimeHarp is less than 25 ps, while the SPADs have a 50 ns dead time. Given these technical specifications, the TimeHarp will continue to adequately serve this FCS instrument through potential future detector upgrades.

3.6 Addition of Second Detection Pathway

Detector afterpulsing introduces systematic error into all FCS measurements. Numerous data analysis strategies have been devised to account for its effects, but
one of the most effective methods to drastically reduce its effects is cross-correlation analysis.\textsuperscript{92} This requires the introduction of a second detection pathway and a second detector. Because afterpulsing is a stochastic process, each APD will have a different contribution to the signal from afterpulsing. These effects do not correlate with one another, meaning that though they may contribute to each detector’s autocorrelation function, they disappear in the cross-correlation function.

The addition of a second detection channel in a Hanbury Brown and Twiss (HBT) configuration was made with the introduction of a non-polarizing beamsplitter cube (10BC17MB.1, Newport Corporation, Irvine, CA) placed after the third emission filter. The beamsplitter transmits about half of the incident photons and reflects the remainder. The confocal detection pathway in the previous instrument scheme (\textit{vide supra}, Section 3.4.2) was duplicated with the same optical and opto-mechanical parts. Care was taken to space each optic at the same distance from the beamsplitter as the corresponding optic in the other pathway so as to prevent spurious correlations. The two-channel design of the TimeHarp makes simultaneous recording of data from the two detection channels straightforward, as shown in Figure 3.11. Alternatively, the APDs can be connected to the MCS cards for confocal scanned imaging. Use of the transillumination pathway on the expanded instrument is illustrated in Figure 3.12 and Figure 3.13.
Figure 3.11: Schematic of the FCS instrument with two confocal detection pathways being used for fluorescence measurements. The APDs are connected to a two-channel hardware correlator for data acquisition.

Figure 3.12: Schematic of the FCS instrument with the imaging pathway engaged by the slide-in mirror and transillumination provided by a 632.8 nm HeNe laser on a dual detection pathway setup.
Figure 3.13: Schematic of the FCS instrument with the transillumination and two confocal pathways used in conjunction with the piezoelectric sample stage to produce confocal scanned images. Either of the dual detection pathways connected to two different multi-channel scaler cards can be used to record the intensity time trace used to create the confocal scanned image.

3.6.1 Hardware Correlator Data

The PicoHarp TCSPC module described in Section 3.5.2 was used to acquire and correlate fluorescence signals from freely diffusing Alexa Fluor® 488 at a concentration of 100 pM in 1X PBS (i.e., 137 mM NaCl, 10 mM Na₂HPO₄, and 2.7 mM KCl) at pH 7.4. The instrument configuration depicted in Figure 3.11 was used to acquire data from both APDs. The autocorrelation curve of each channel and the cross-correlation curve between the two channels were acquired simultaneously in real-time. Figure 3.14 shows five runs for each with their averages. Averages of five runs showed very high precision and repeatability that was much better than that obtained from multiple runs using the MCS cards for acquisition. A likely reason is the larger number of data points collected.
and analyzed with the hardware correlator. The effect of afterpulsing is clearly apparent in the autocorrelation curves for both pathways with a fast decay at $\tau = 10^{-7}-10^{-6}$ s with high amplitude. The fast decay is eliminated in the cross-correlation curves where only the decay at $\tau = 10^{-4}-10^{-3}$ s is present. This further suggests that the fast decay was due to afterpulsing. Figure 3.15 shows the averages of the three sets on a single plot for easier comparison. The amplitude of the cross-correlation roughly matches that of the autocorrelation curve with lower amplitude, consistent with the fact that the cross-correlation between the two detectors can never exceed the magnitude of the correlation of either detector with itself. The five-run average of the cross-correlation curve was fit using the 3D-diffusion model in Equation 1.6, which very adequately describes the entirety of the curve with a $\chi^2$ value of approximately $10^{-2}$. Though the $D$ value of $2.38 \times 10^{-7}$ cm$^2$/s significantly deviates from the literature value of $4.35 \times 10^{-6}$ cm$^2$/s, the tight fit of the diffusion model to the experimental data indicates that probe volume shape is reasonable, but significantly larger than expected. More accurate positioning of the confocal pinholes, as was achieved before acquiring the figures in Section 4.6, would have likely produced a smaller probe volume and a $D$ value closer to that reported in the literature.
Figure 3.14: PicoHarp-acquired correlation curves of fluorescence intensity fluctuations from 100 pM Alexa Fluor 488® in 100 pM in 1X PBS at pH 7.4; autocorrelation curves for (a) channel 1 and (b) channel 2, and (c) cross-correlation curves between the detection channels. Point-by-point averages of the five runs were performed to produce the overlaid blue curves.
Figure 3.15: Five-run averages of the PicoHarp-acquired autocorrelation and cross-correlation curves; replotted from Figure 3.14.

Figure 3.16: Five-run average of the PicoHarp-acquired cross-correlation curves from Figure 3.14(c) with the corresponding 3D-diffusion model fit obtained with Equation 1.6.
3.7 Switch to 458 nm Excitation Laser

Initial attempts to measure fluorescence from a bulk solution of FMN using the instrument configuration depicted in Figure 3.11, even in the low micromolar range, produced Poisson distributions of intensity. This result was unsurprising considering that FMN absorbs weakly at 488 nm. One of FMN’s two strong absorption peaks falls near the 458 nm laser line. A 458 nm solid-state continuous laser (Sapphire 458 LP, Coherent, Inc., Santa Clara, CA) with the same physical form factor and beam shape specifications was used to replace the 488 nm laser, as is schematically illustrated in Figure 3.17. A substantial increase in fluorescence signal resulting from FMN illuminated at this wavelength was observed.

Figure 3.17: Schematic of the FCS instrument with 458 nm excitation and with two confocal detection pathways being used for fluorescence measurements. The APDs are connected to a two-channel hardware correlator for data acquisition.
3.8 Sample Drift Diagnosis and Reduction

A major experimental challenge for reliable data acquisition was drift of the sample out of the optical probe volume. Several approaches were taken to diagnose and reduce this drift. First, the assembled device was mounted securely in the slide mount. Beyond the tightening screws on the mount, aluminum blocks weighing either 50 g or 100 g were placed on top of the device, and brass shims were inserted along the slide mount’s translation axis to limit θx and θy rotations. The stability of the piezoelectric stages was confirmed by monitoring their reported position over time. Two different objectives with the same optical specifications were used with no discernable difference in drift. Repeated acquisitions over a 5 hr period reported continual drift, meaning that the sample did not settle to an equilibrium position. Finally, two environmental factors were controlled. A dehumidifier was used to set the room at a stable humidity of approximately 40% RH. Temperature gradients were lessened by isolating the laser source from the rest of the excitation pathway and rerouting HVAC away from the instrument.

In Figure 3.18, typical data acquired under a large temperature gradient show the effect of the ZMW drifting out of the probe volume. Figure 3.18(a) shows how quickly a drift response can begin and Figure 3.18(b) shows the large difference in signal fluctuations pre-drift and post-drift. In both cases, post-acquisition refocusing showed that the sample drifted out-of-focus along all three axes. Although post-acquisition data analysis techniques can baseline correct the data to a limited extent, it is clear that the variability of the data quickly and steadily decreases as the overall intensity decreases.
Most of the signal spikes are due to large fluorescent bursts. As the frequency and magnitude of these spikes decrease, the chemical information from the experiment decreases dramatically. The data acquired under a small temperature gradient shows an approximately 45 min period of signal stability before a steady intensity decrease. Unfortunately, signal stability for that length of time was rare.
Figure 3.18: Fluorescence signal drift due to sample drift. (Top) A quick drift response compared to a long stable period; (bottom) short time of stability followed by a rapid drift compared to the same long stable period. The insets show the first 10 min of the large temperature gradient data.
3.9 Concluding Remarks

The upgrades described here to the foundational FCS optical system presented in Chapter 2 represent a substantial expansion of analytical capabilities. The experimental protocols and techniques that were developed and refined to utilize those capabilities enable measurements to be made with high spatiotemporal resolution. Utilization of the upgraded instrument is demonstrated for spectroelectrochemical study of freely diffusing flavin mononucleotide (FMN) in Chapter 4.
CHAPTER 4:

SPECTROELECTROCHEMISTRY OF FREELY DIFFUSING FLAVIN MONONUCLEOTIDE

This chapter is based, in part, on previously published material from:


4.1 Introduction

The fluorescence correlation spectroscopy (FCS) optical microscope described in Chapter 3 constitutes a powerful tool for the study of fluorescence fluctuations at the nanometer scale and with temporal resolution down to 1 ns. Following up on previous studies utilizing zero-mode waveguides (ZMWs) with wide-field observation, single pore investigations were pursued at the higher spatiotemporal resolution enabled by the confocal system.

While many nanochannel device configurations provide optical access to the entire channel length, they are difficult to fabricate with multiple sub-100 nm dimensions. As such, additional assumptions must be made when using them to multiplex nanostructure arrays. Nanopores are readily available with multiple sub-100 nm dimensions from track-etched membranes, focused ion beam (FIB) milling, or electron beam lithography and reactive ion etching. As the long-axis of nanopores is
typically aligned along the optical axis, the depth into the pore that can be observed optically is limited. Thus, careful experimental design is necessary to study transport and reaction in the observable region of a nanopore.

Wide-field imaging has been and will continue to be a powerful tool for the study of arrays of nanopores, whereas, confocal microscopy affords similar information at higher spatial resolution and much higher temporal resolution. Where wide-field imaging often requires at least one species in a chemical system to be immobilized to the bottom of a nanopore to ensure sufficient residence time to produce a reliable signal, the temporal resolution available on the instrument described in Chapter 3 enables measurement and study of processes that occur on the microsecond timescale, such as diffusion of small molecules.

The study of freely diffusing, rather than immobilized, molecules allows for longer measurement times, because fluorescent molecules continuously diffuse into and out of the observation volume. Even if a fluorescent molecule is photobleached, it can be replaced by other fluorescent molecules. This flexibility enables a number of experiments using perturbations to the chemical system to be studied, whereas, immobilized fluorophores are less robust, due to photobleaching.

4.2 Bulk Electrochemical Measurements of Flavin Mononucleotide (FMN)

Bulk electrochemical data were obtained to form a basis for single-molecule spectroelectrochemical experiments. Cyclic voltammograms (CVs) were collected with a 100 mV/s scan rate on 5 mM flavin mononucleotide (FMN) (F8399, Sigma-Aldrich
Corporation, St. Louis, MO) in 100 mM of five different pH buffers. These were pH 2.90 and 4.80 citrate buffers, pH 6.90 phosphate buffer, pH 8.85 borate buffer, and pH 10.85 carbonate buffer. A 2 mm disc working electrode (WE), a Pt counter electrode (CE), and a Ag/AgCl reference electrode (RE) were used to measure the CVs. Figure 4.1 shows the CVs at each potential and their corresponding half-wave potentials, calculated by,

\[
E_{1/2} = \frac{(E_{\text{peak,cathodic}} + E_{\text{peak,anodic}})}{2}
\]  

(4.1)

The trend of \(E_{1/2}\) values decreasing with increasing pH was observed previously with similar values.\(^6\) At pH 2.90, the \(E_{1/2}\) value determined by Equation 4.1 is -0.189 V vs. Ag/AgCl. This value was used in single-molecule spectroelectrochemical experiments at the same pH (vide infra, Sections 4.3, 4.8, 4.9, and 4.10).

Figure 4.1: Comparison of cyclic voltammograms for 5 mM FMN in five different pH buffers, each at 100 mM. The vertical dashed lines denote the value of \(E_{1/2}\) determined for each pH.
4.3 Bulk Spectroelectrochemistry of FMN on Indium Tin Oxide (ITO)

4.3.1 ITO Device for Bulk Spectroelectrochemistry

Bulk measurements are commonly used as a control system in FCS studies. Indium tin oxide (ITO) coated soda-lime coverslips (06488-AB, SPI Supplies, West Chester, PA) make bulk spectroelectrochemistry studies straightforward to perform. The conductive ITO layer has a sheet resistance of 15-30 Ω/square, adds 350 nm to the thickness of a 170 μm thick No. 1.5 coverslip, and is more than 85% optically transparent. The ITO-coated coverslip was adhered with double-sided, high dielectric constant Kapton® Polyimide Tape (16087-25, Ted Pella, Inc., Redding, CA) to a glass slide with two pre-drilled access holes. The glass slide provided rigidity, reducing breakage and preventing major flexure of the device during experiments. A PDMS well was plasma-treated and then thermally bonded to the glass slide over one of the glass wells. A working electrode (WE) was soldered to the ITO surface through the other access hole in the glass slide.

4.3.2 Spectroelectrochemical Measurements

Fluorescence measurements were performed using the instrument shown in Figure 3.17 with the beamsplitter cube removed to maximize the signal reaching APD in detection pathway 1. A sample of 6.53 nM FMN in 100 mM citrate buffer at pH 2.90 was illuminated with 40 μW power in the plane at the ITO/liquid interface. The concentration of FMN corresponds to an average molecular occupancy, \( <N> \approx 1.2 \) molecules/probe volume. Electrochemical potential modulation and measurements
were made using a potentiostat (750E, CH Instruments, Austin, TX). A Pt counter electrode and Ag/AgCl reference electrode were inserted into the PDMS well containing the sample solution. The ITO surface was used as the working electrode (WE).

4.3.3 Bulk Spectroelectrochemistry Results

4.3.3.1 Fluorescence Coupled to Static Electrochemistry

Fluorescence emission rates of freely diffusing FMN molecules under static potential control were measured in 50 mV increments above and below $E_{1/2} = -0.189$ V vs. Ag/AgCl in the range $E_{appl} = +0.061$ V to -0.439 V. At each new $E_{appl}$, the potential was held for $\approx 30$ s to ensure that a steady-state was reached at the ITO electrode before the fluorescence signal was recorded. The fluorescence signal intensity was recorded using the TimeHarp and collected in 1 ms time bins for analysis. Histograms of the intensity traces at each potential were created, and Poisson functions were fit to the histograms to produce the distributions shown in Figure 4.2. As a general trend, the intensity distribution shifts toward lower intensity values at more negative potentials as the fraction of reduced, less emissive molecules in the population increased. This is consistent with the redox scheme seen in Figure 1.1. The intensity at the most oxidative $E_{appl}$, +0.061 V, was recorded both first and last to ensure that other factors such as photobleaching did not significantly affect the observed trend; data from the latter acquisition are not shown. Figure 4.3 plots the expectation values from the Poisson fits as a function of $E_{appl}$. With the exception of the two most negative $E_{appl}$, the trend is
monotonic, suggesting that the signal observed at $E_{\text{appl}} = -0.339$ V and below is the background.

![Diagram with histograms and Poisson distribution fits](image)

Figure 4.2: Poisson distribution fits to histograms of fluorescence intensities at different values of $E_{\text{appl}}$ for 6.53 nM FMN in 100 mM citrate buffer at pH 2.90. Experimental data are included with the fit for $E_{\text{appl}} = +0.061$ V to illustrate the quality of the fit. The arrow denotes the order in which the data were acquired.
4.3.3.2 Fluorescence Coupled to Dynamic Electrochemistry

The data obtained at static oxidative and reductive potentials establish the trend of fluorescence with applied potential as a foundation to investigate the fluorescence response under time-varying potential control. Whereas, static potential acquisitions were performed after the electrode surface reached steady-state, active potential control acquisitions were started simultaneously by a voltage trigger sent from the hardware correlator to the potentiostat. Because FMN is reoxidized by molecular oxygen in solution between runs, active potential runs were initiated at the most oxidative potential to provide a more consistent starting point.
The result of a single-molecule occupancy chronofluorometry experiment is shown in Figure 4.4. This experiment is the fluorescence analog of chronoamperometry, monitoring the fluorescence, as opposed to the current, response of a redox system to alternating potential steps. Following from Figure 4.2, the same ± 0.25 V overpotential range vs. Ag/AgCl ($E_{app} = +0.061$ V and -0.439 V) was used to produce conditions where FMN can be fully oxidized or fully reduced. The fluorescence intensity at the initial relatively positive potential is nearly constant at $I_{ox} \approx 34 \text{ ms}^{-1}$, but it sharply decreases once the more negative potential is applied, reaching a near constant fluorescence signal at $I_{red} \approx 27 \text{ ms}^{-1}$ during each of the six reductive steps. Surprisingly, once the potential is returned to an oxidizing value, the fluorescence signal sharply drops before steadily climbing to a peak $I_{ox} \approx 31 \text{ ms}^{-1}$ in the last five oxidative steps. The sharp drop in fluorescence accompanying the rising potential edge may be due to a partially-reduced FMN/FMNH$_2$ intermediate following a different redox pathway back to the oxidized form (vide infra), one that passes through an intermediate with a lower quantum efficiency. Microscopic reversibility would suggest that a change in the preferred pathway with direction is unlikely; the conditions for which this may be possible are described in detail in Section 4.11. The consistent trends in the fluorescence signal in the last five cycles demonstrates the repeatability of the redox system.
Figure 4.4: Chronofluorometry of 6.53 nM FMN in 100 mM citrate buffer at pH 2.90 at an ITO working electrode. The potential (vs. Ag/AgCl) is modulated at 5 s intervals between +0.061 V and -0.439 V, and fluorescence emission is monitored from the ITO/liquid interface, the data being binned to 10 ms. The potential program is shown at the top, and the fluorescence response at the bottom. A 21-point boxcar smoothing routine was applied to the raw data to produce the overlaid black curve.

Data from the fluorescence analog of cyclic voltammetry experiments are shown in Figure 4.5, Figure 4.6, and Figure 4.7. The fluorescence signal for the fastest scan rate, 0.5 V/s, shown in Figure 4.5, is indistinguishable from noise. The smoothed version of the data reveals some periodicity in the fluorescence signal, but without a strong relationship to the applied potential. Along with the observation that $<I>$ never returns to the initial value, the lack of a clear dependence on $E_{appl}$ shows that the process is diffusion-limited at this scan rate. Figure 4.6 shows data acquired at an intermediate scan rate of 50 mV/s. Similar to the data in Figure 4.4, fluorescence is strongly
correlated with the applied potential, but is out-of-phase by approximately 2 s at the
oxidative peak and approximately 5 s at the reductive trough. An intermediate redox
state is possibly observed during the reductive sweep, where an increase in
fluorescence signal is observed as a shoulder at approximately the most negative
potential. Lastly, Figure 4.7 shows data from the slowest scan rate, 1 mV/s. It is the only
dynamic potential control experiment here that exhibits a near complete recovery of
the initial fluorescence signal by allowing adequate time for the diffusion of the majority
of the reduced molecules to the ITO electrode surface. Once again, the fluorescence
signal is not monotonic with the direction of the potential sweep. Shortly before the
reductive trough is reached, the fluorescence peak is observed. The increases in
fluorescence intensity at the most negative potentials, apparent in Figure 4.6 and Figure
4.7, are unexpected. The relationship between the redox state and the quantum
efficiency (vide supra, Section 1.5.2) is one of the foundational assumptions for this
work. This relationship holds elsewhere, notably, in Figure 4.2, Figure 4.3, and the data
discussed in Section 4.10. A number of explanations can be posited to reconcile this
observation and are discussed in more detail in Section 4.11 when compared to data
from ZMW experiments.
Figure 4.5: Cyclic potential sweep fluorescence measurements of 6.53 nM FMN in 100 mM citrate buffer at pH 2.90 at a scan rate 0.5 V/s at an ITO working electrode. Fluorescence emission is monitored from the ITO/liquid interface, the data being binned to 10 ms. The potential program is shown at the top, and the fluorescence response at the bottom. A 21-point boxcar smoothing routine was applied to the raw data to produce the overlaid black curve.
Figure 4.6: Cyclic potential sweep fluorescence measurements of 6.53 nM FMN in 100 mM citrate buffer at pH 2.90 at a scan rate 0.05 V/s at an ITO working electrode. Fluorescence emission is monitored from the ITO/liquid interface, the data being binned to 100 ms. The potential program is shown at the top, and the fluorescence response at the bottom. A 21-point boxcar smoothing routine was applied to the raw data to produce the overlaid black curve.
Figure 4.7: Cyclic potential sweep fluorescence measurements of 6.53 nM FMN in 100 mM citrate buffer at pH 2.90 at a scan rate 0.001 V/s at an ITO working electrode. Fluorescence emission is monitored from the ITO/liquid interface, the data being binned to 100 ms. The potential program is shown at the top, and the fluorescence response at the bottom. A 21-point boxcar smoothing routine was applied to the raw data to produce the overlaid black curve.

4.4 Zero-Mode Waveguide (ZMW) Array Fabrication

ZMW arrays were fabricated using an established process\textsuperscript{32-33} with process modifications appropriate to the experiments described here. Electron beam evaporation was used to deposit 5 nm of Ti and 200 nm of Au onto borosilicate coverslips (No. 1.5H, Schott North America, Inc., Elmsford, NY) in a patterned geometry. Although Al is often used as the optical cladding layer for ZMW arrays due to its large skin depth, Au was chosen, because it has a large ideally polarizable region and is thus more amenable to electrochemical experiments. Focused ion beam (FIB) milling was
used to create 11 x 11 square ZMW arrays typically of 5 μm pitch, for side lengths of 50 μm. All of the pores in each array were milled in the same run at 9.7 pA with a dwell time of 0.1 ms to produce pores that effectively confine the optical field and reduce the observation volume. The four corner pores of the 11 x 11 arrays used here were milled a second time to enlarge them to about twice the diameter of the other pores. Along with four FIB milled microchannels offset from the array, these larger corner pores were used as fiducial marks to aid in locating the ZMW array with the transillumination imaging pathway. Figure 4.8 shows scanning electron micrographs (SEMs) of a typical array with magnified views. A large corner pore is shown in Figure 4.8(b). A cross-section of a pore in Figure 4.8(d) reveals the conical frustum shape obtained with FIB-milling of ZMWs, as well as the over-etched region commonly occurring in FIB-milled pores.\(^\text{32}\) This is in contrast to the cylindrical shapes obtained using electron beam lithography, which produces no over-etch.\(^\text{23}\) The over-etched region extends past the Ti/glass interface less than 50 nm in well-controlled milling processes and typically displays the same width as the pore bottom diameter, \(d_{\text{bottom}}\).
Figure 4.8: Scanning electron micrographs of a ZMW array at different magnifications and perspectives, (a) top-down view of the entire 11 x 11 ZMW array, (b) top-down view of a 3 x 3 subset in the top-right corner of the same array, (c) top-down view of a single nanopore with $d_{\text{top}} \approx 100$ nm and $d_{\text{bottom}} \approx 50$ nm, and (d) cross-section of a nanopore filled with Pt to assist imaging.
After FIB milling, the devices were cleaned with 1.0 M KOH at 80°C for 45 min, concentrated H$_2$SO$_4$ for 10 min, and concentrated HNO$_3$ for 10 min, followed by a DI water rinse. As above (*vide supra*, Section 4.3.1), the device was adhered with Kapton® tape to a glass slide with pre-drilled access holes for the Au pad containing the ZMW array and the Au pad used to attach the working electrode (WE). The glass slide provided rigidity, reducing breakage and preventing significant deformation of the device during the experiment. A PDMS well was plasma treated and then thermally bonded to the glass slide over the glass well surrounding the ZMW array. A working electrode was soldered to the other Au pad through the other hole in the glass.

A schematic illustration of a ZMW with an optical field incident from below is shown in Figure 4.9. The schematic is analogous to the cross-section of a pore seen in Figure 4.8. Four molecular states of FMN are represented. Fully reduced FMNH$_2$ molecules do not emit significant fluorescence, even under the most intense illumination at the bottom of the over-etched region. Fully oxidized FMN molecules near the bottom of the pore, or in the over-etched region, fluoresce strongly. Oxidized molecules, in a portion of the ZMW where the optical field has significantly decayed, are excited and fluoresce weakly as well. Finally, oxidized molecules near the ZMW/bulk solution interface, where the optical field has been attenuated by multiple orders of magnitude, are not excited and do not emit to any meaningful degree.
Figure 4.9: Schematic illustration of an over-etched ZMW with reduced and oxidized FMN molecules shown freely diffusing. The decay of the optical field is represented by the green-to-white gradient in the pore volume.

4.5 ZMW Optical Profile Simulations

Finite element simulations using COMSOL (COMSOL Multiphysics® Version 4.4, Inc., Burlington, MA) were performed over a two-dimensional domain representing the cross-sectional geometry of the zero-mode waveguides employed in these experiments. The basic geometry was determined from SEMs of pore cross-sections, such as Figure 4.8(d). Structures exhibiting various values for $d_{\text{top}}$, $d_{\text{bottom}}$, and $d_{\text{top}}$:$d_{\text{bottom}}$, were simulated. The simulations used the Electromagnetic Waves, Frequency Domain package within the Wave Optics Module of COMSOL. A large domain above and below the pores was used to eliminate boundary interference. The mesh was refined within and just above the single pore to provide sufficient resolution in the region of interest.
Selected simulation results are shown in Figure 4.10. Each of the three pores shown has a ratio $d_{\text{top}}:d_{\text{bottom}} = 2$ and identical incident optical fields. Other simulations with either constant $d_{\text{top}}$ or $d_{\text{bottom}}$ and various values of $d_{\text{top}}:d_{\text{bottom}}$ revealed that $d_{\text{bottom}}$ is the most important variable determining the optical field attenuation, which is sensible, because the bottom pore opening serves as an aperture for light entering the pore. In the $d_{\text{bottom}} = 40$ nm pore, the optical field is damped to 10% of the incident intensity at approximately 42 nm above the Ti/glass interface. In the $d_{\text{bottom}} = 60$ nm pore, damping to 10% is not reached until approximately 66 nm above the Ti/glass interface.
Figure 4.10: Cross-sectional heat maps of evanescent field amplitudes in conical nanopores obtained by finite element simulations, showing attenuation as a function of nanopore geometry. All three structures exhibit $d_{top}:d_{bottom} = 2$ with $d_{bottom} = (a) 40$ nm, (b) 50 nm, and (c) 60 nm, and all three are plotted using the intensity scale shown adjacent to panel (b).
Figure 4.11 takes these optical field attenuation calculations further by determining the effective observation volume as a function of $d_{\text{bottom}}$ for different $d_{\text{top}}$: $d_{\text{bottom}}$. Optical profile decay data from additional simulations were analyzed to determine the depth into the pore at which the intensity had decayed to $1/e^2$ of its initial value, or an attenuation of 8.69 dB. This attenuation corresponds to a weak evanescent field strength that is unlikely to cause significant excitation of fluorescence. Using the pore diameter corresponding to that depth, the depth itself, and $d_{\text{bottom}}$, the volume of the conical frustum was calculated for each size and diameter ratio, yielding the effective observation volume inside of the pore. To match the experimental geometry, the volume of the over-etched region was also included. It was approximated, as described above in Section 4.4, as a cylinder with a diameter equal to $d_{\text{bottom}}$ of the pore and a depth of 50 nm. Figure 4.11 compares the $V_{\text{eff}}$ curves for $d_{\text{top}}$: $d_{\text{bottom}}$ ratio of 1.5, and the inset shows that for small values of $d_{\text{bottom}}$, $V_{\text{eff}}$ is dominated by the over-etched volume contribution, for example, constituting 66% of the pore volume at $d_{\text{bottom}} = 30$ nm. The contributions of the pore and over-etched volumes become equal at $d_{\text{bottom}} \approx 54$ nm for $d_{\text{top}}$: $d_{\text{bottom}} = 1.5$. The inverse relationship between $V_{\text{eff}}$ and the concentration at which the expectation value of molecular occupancy reaches unity, (i.e., single occupancy, $<N> = 1$), demonstrates that concentrations as high as 30 μM can be used with the lowest value of $d_{\text{bottom}}$ shown here, while still achieving single occupancy conditions.
Figure 4.11: Effective volumes (left ordinate) and corresponding concentrations for single-molecule occupancy (right ordinate) for \(d_{\text{top}}:d_{\text{bottom}}\) ratios of 1.5, 2.0, and 3.0 as a function of \(d_{\text{bottom}}\). (Inset) The effective volumes without an over-etched region (left ordinate) and corresponding concentrations (right ordinate) molecule occupancy for \(d_{\text{top}}:d_{\text{bottom}}\) ratio of 1.5.

The ZMWs used here have \(d_{\text{bottom}} \approx 50\) nm, corresponding to a total effective volume of approximately 200 zL, as is seen in Figure 4.11. Monte-Carlo simulations of Brownian motion were performed in the same geometries as in Figure 4.10. The mean residence time was defined as the time it takes for a particle randomly positioned inside of the effective volume of the pore to diffuse out. The simulations calculated the mean residence time to be \(\approx 0.5-3.5\) μs, depending on the pore geometry.
4.6 Confocal Scanning of ZMW Arrays

Immediately after the ZMW device was cleaned (*vide supra*, Section 4.4), it was filled with 10 μM FMN in 100 mM citrate buffer at pH 2.90. This concentration corresponds to a molecular occupancy of \(<N> = 1.2\) in the 200 zL observation volume, the same as the bulk experiments detailed in Section 4.3.3. Repeated trials proved that the cleaning process provided sufficient wettability for the pores to fill by capillary action. The confocal scanning protocol detailed in Section 3.4.2 was used to locate a ZMW array of interest. Figure 4.12 shows a transillumination confocal scanned image of the 11 x 11 ZMW array used for spectroelectrochemical experiments detailed below. Only the four fiducial corner pores are observed, as the smaller pores effectively attenuate the transillumination radiation. An epi-illumination fluorescence confocal scanned image of the array is shown in Figure 4.13. Approximately 60% of the pores are clearly illuminated by fluorescence from FMN, and within this set, there is a distribution of intensities. Not surprisingly, the four corner fiducial pores are the brightest.
Figure 4.12: Transillumination confocal scanned image of a ZMW array made of $d_{\text{top}} \approx 100$ nm, $d_{\text{bottom}} \approx 50$ nm nanopores filled with 10 μM FMN in 100 mM citrate buffer at pH 2.90. FMN does not fluoresce at the incident wavelength used to produce this image.
Figure 4.13: Epi-illumination fluorescence confocal scanned image of the same ZMW array shown in Figure 4.12. Nanopores are filled with 10 μM FMN in 100 mM citrate buffer at pH 2.90, which is excited at 458 nm and emits, locating the individual ZMW nanopores.
4.7 Colocalization of Optical Probe Volume and ZMWs

A bright pore in the middle of the array was chosen for spectroelectrochemical investigation. The x and y coordinates were recalled from an indexed image. The fluorescence signal intensity was monitored as the x and y positions of the sample and the z position of the objective were iteratively stepped in 100 nm increments. Once the fluorescence signal was maximized, the optical probe volume was considered to be colocalized with the ZMW. This was confirmed by stepping the potential to a reductive value and back to an oxidative value, and watching the fluorescence signal drop and then return.

4.8 Constant Potential Scans in ZMWs

In common with the ITO experiments, the emission rates of freely diffusing FMN molecules were characterized first under static potential control in the range $E_{\text{appl}} = +0.061 \text{ V}$ to $-0.439 \text{ V}$ at FMN concentrations of 10 μM and 1 μM, corresponding to $<N> = 1.2$ and 0.12 molecules, respectively. Solutions were held at constant potential for $\approx 30 \text{ s}$ in order to ensure a steady-state current had been reached at the ZMW electrode, after which the fluorescence signal intensity was recorded using the TimeHarp and collected in 1 ms time bins for analysis. Histograms of the intensity traces at each potential were created, and Poisson functions were fit to the histograms to produce the distributions shown in Figure 4.14. At both average occupancy levels, the distribution shifts to higher intensity values as the potential is made more positive, consistent with the molecules being more likely to be in the oxidized state, FMN in Figure 1.1.
Figure 4.14: Poisson distribution fits to histograms of fluorescence intensities at different values of $E_{\text{appl}}$ for (a) 10 μM and (b) 1 μM FMN in 100 mM citrate buffer at pH 2.90. Experimental data are included with the fit for $E_{\text{appl}} = +0.061$ V to illustrate the quality of the fit. The arrow denotes the order in which the data were acquired.
At the most positive value, $E_{appl} = +0.061 \text{ V}$, the peak of the fluorescence emission distribution occurs at $I_{ox} \approx 64 \text{ ms}^{-1}$. At the most negative value, $E_{appl} = -0.439 \text{ V}$, the fluorescence signal decreases to $I_{red} \approx 41 \text{ ms}^{-1}$. The intensity observed at oxidizing potentials is consistent with estimates of the emission rate for FMN excited at 458 nm based on single-molecule occupancy, the measured irradiance at the ZMW array, and the estimated collection efficiency. However, the “dark state” emission observed at $E_{appl} = -0.439 \text{ V}$ is significantly higher than the apparent emission level, $I_{bkgnd} \approx 3 \text{ ms}^{-1}$, observed in the absence of fluorophores in control experiments. The latter observation can be understood by considering three dynamic processes: (1) the intrinsic photopumping and luminescence dynamics of the fluorophore, (2) transport of both oxidized and reduced molecules into and out of the ZMW nanopore, and (3) heterogeneous electron transfer at the Au-cladding/working electrode. The intrinsic photopumping and emission dynamics are overwhelmingly associated with the oxidized state. Thus, the most likely explanation for the observation that $I_{red} > I_{bkgnd}$ involves occupation of the ZMW nanopore by oxidized FMN under nominally reducing conditions. One source of oxidized FMN is from the background population that exists since the beginning of the experiment, which can diffuse into the excitation volume. Additionally, FMNH$_2$ may react with dissolved O$_2$ in the nanopore to produce FMN, independent of the potential on the electrode. Some of these FMN molecules will diffuse to the Au surface and be reduced prior to diffusing out of the pore, so the level of emission observed at $I_{red}$ likely reflects the balance between: (1) new oxidized species diffusing into the pore, (2) new oxidized species being generated in the pore, and (3)
oxidized species being reduced while in the ZMW. Since the time bin for data collection (i.e., 1 ms) is \( \geq 300 \) times the average residence time obtained from Monte Carlo simulations in Section 4.5, there is ample time to sample many individual molecules during the collection window.

The narrowing of the intensity distribution at lower concentration provides another interesting feature of these data. When the static potential experiment is repeated at [FMN] = 1 μM, shown in Figure 4.14(b), the distribution shifts to higher intensity values, as the potential is made more positive. This also happens with the [FMN] = 10 μM data in Figure 4.14(a), but the spread of fluorescence intensity distributions is considerably narrower at the lower concentration. The negative end of the distribution decreases to \( I_{\text{red}} \approx 34 \text{ ms}^{-1} \), but the positive end at \( E_{\text{appl}} = +0.061 \text{ V} \) has a maximum at \( I_{\text{ox}} \approx 40 \text{ ms}^{-1} \). The narrowed distribution is also consistent with the mechanism for \( I_{\text{bkgnd}} \) proposed above, as the lower concentration would be expected to yield a smaller departure from the background simply due to the smaller number of oxidized species capable of diffusing into the ZMW.

4.9 Chronofluorometry: Potential Step Scans in ZMWs

Having established the conditions under which single-molecule occupancy is achieved in ZMWs, the optimum geometries for coupling optical radiation into solutions supporting single redox-active molecules in close proximity to a working electrode, and the static potential dependence of FMN emission from ZMWs, the next fluorescence measurements were made with time-varying potentials to explore how
electrochemistry couples to a population of freely diffusing single-molecules. Figure 4.15 shows a comparison of the potential dependence of FMN luminescence from a single ZMW nanopore obtained with 10 μM FMN in 100 mM citrate buffer at pH 2.90 to the bulk CV at the same pH shown in Figure 4.1. Even though the luminescence data are acquired in a very different physical environment, the inflection in the potential-dependent luminescence curve, at -0.115 V, is in reasonable agreement with the bulk electrochemical value, is $E_{1/2} = -0.189$ V vs. Ag/AgCl.

Figure 4.15: Comparison of the potential dependence of FMN luminescence (discrete data points) from a single ZMW nanopore obtained at an FMN concentration of 10 μM in 100 mM citrate buffer at pH 2.90 to a bulk measurement of the cyclic voltammogram for 5 mM FMN in the same buffer (solid curve). The curved dashed line gives the fit of the luminescence data to a sigmoidal optical response function. The vertical dashed lines denote the value of $E_{1/2}$ determined by each method; these values are -0.115 V for the ZMW and -0.189 V for bulk.
A single-molecule occupancy chronofluorometry experiment is shown in Figure 4.16. Analogous to the more familiar chronoamperometry, this experiment monitors the fluorescence, as opposed to the current, response of a redox system to alternating potential steps. Given the $E_{1/2} = -0.189$ V determined for FMN in Figure 4.14, the chronofluorometry experiment was designed to step between ± 0.25 V overpotentials vs. Ag/AgCl ($E_{appl} = +0.061$V and -0.439V) in order to produce conditions in which FMN alternates between the fully oxidized and fully reduced states, represented in Figure 1.1 as FMN and FMNH$_2$. FMNH$_2$ is the fully protonated form of reduced FMN, appropriate to experiments at pH 2.90. Although both longer and shorter time windows were investigated, the 5 s potential steps used in Figure 4.16 are sufficiently long to allow the system to achieve steady-state at both oxidizing and reducing conditions.
Figure 4.16: Chronofluorometry of 10 μM FMN in 100 mM citrate buffer at pH 2.90. The potential (vs. Ag/AgCl) is modulated at 5 s intervals between +0.061 V and -0.439 V, and fluorescence emission is monitored from a single $d_{\text{top}} = 100 \text{ nm}$, $d_{\text{bottom}} = 50 \text{ nm}$ ZMW nanopore using 10 ms time bins. The smoothed bulk data from Figure 4.4 are shown for comparison in the middle of the potential program at the top and the fluorescence response at the bottom.
The fluorescence response in Figure 4.16 is strongly correlated to the applied potential steps. Stepping to a reducing potential from the initial oxidizing potential causes an immediate decrease in emission intensity from $I_{\text{ox}} \approx 64 \text{ ms}^{-1}$ to $I_{\text{red}} \approx 41 \text{ ms}^{-1}$, consistent with the steady-state values shown in Figure 4.14. Interestingly, when the potential is returned to an oxidizing value, the fluorescence signal exhibits a significant and reproducible transient spike before decaying to the steady-state value $I_{\text{ox}} \approx 64 \text{ ms}^{-1}$. A transient spike is not observed in the raw data that formed Figure 4.14, because the static potential results were obtained at steady-state. Because the potential step occurs rapidly compared to molecular transport, any reduced molecules in the nanopore would be oxidized within the time required to diffuse to the pore wall, $\tau_D \approx 1.5 \mu$s for a pore with $d_{\text{bottom}} = 60 \text{ nm}$ and molecules with $D \approx 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. Thus, a momentarily larger population of oxidized FMN in and near the ZMW pores is established and partially responsible for the sharp increase in fluorescence observed on the positive potential step. The balance between diffusion of FMN into the pore and oxidation of FMNH$_2$, both contributing positively to the fluorescence signal, and diffusion of FMN out of the pore and photobleaching, both contributing negatively to the fluorescence signal, would serve to equilibrate to a steady-state population.

4.10 Fluorescence-Reported Cyclic Potential Sweeps in ZMWs

The single-molecule occupancy fluorescence analog of cyclic voltammetry experiments is shown in Figure 4.17, Figure 4.18, and Figure 4.19 at three distinct scan rates. At the fastest scan rate, 0.5 V/s, the fluorescence behavior of 10 µM FMN is
similar to that expected from a bulk measurement. As shown in Figure 4.17, the fluorescence response is symmetric and reproducible, oscillating between values of $I_{ox} \approx 70 \text{ ms}^{-1}$ and $I_{red} \approx 43 \text{ ms}^{-1}$, similar to the results obtained at static potentials in Figure 4.14 and with chronofluorometry in Figure 4.16. At an order of magnitude lower scan rate of 50 mV/s, the response develops an asymmetric shape, shown in Figure 4.18, with a shoulder on the negative-going scan and a constant intensity observed over an extended window of reducing potentials. Data from the slowest scan rate studied, 1 mV/s, is shown in Figure 4.19. The potential-dependent fluorescence develops distinct additional states with intermediate fluorescence emission intensities of $I_{int} \approx 32 \text{ ms}^{-1}$ between the $I_{ox} \approx 44 \text{ ms}^{-1}$ and $I_{red} \approx 25 \text{ ms}^{-1}$. 
Figure 4.17: Cyclic potential sweep fluorescence measurements of 10 μM FMN in 100 mM citrate buffer at pH 2.90 at a scan rate 0.5 V/s. The smoothed bulk data from Figure 4.5 are shown for comparison in the middle of the potential program at the top and the fluorescence response at the bottom.
Figure 4.18: Cyclic potential sweep fluorescence measurements of 10 μM FMN in 100 mM citrate buffer at pH 2.90 at a scan rate 0.05 V/s. The smoothed bulk data from Figure 4.6 are shown for comparison in the middle of the potential program at the top and the fluorescence response at the bottom.
Figure 4.19: Cyclic potential sweep fluorescence measurements of 10 μM FMN in 100 mM citrate buffer at pH 2.90 at a scan rate 0.001 V/s. The smoothed bulk data from Figure 4.7 are shown for comparison in the middle of the potential program at the top and the fluorescence response at the bottom.

The asymmetric fluorescence potential response at 50 mV/s and the intermediate emission intensity states observed at 1 mV/s are likely due to equilibriums at intermediate species between FMN and FMNH₂, depicted in Figure 1.1. Though a preferred reaction pathway was denoted, two additional reaction pathways exist with a total of 5 possible intermediates, including 3 semiquinones. At pH 2.90, the reduction of FMN to FMNH₂ is thought to occur through two separate proton-coupled (1e⁻/1H⁺) steps through an intermediate flavosemiquinone (i.e., FMNH⁺ in Figure 1.1) that is stabilized in
this environment. In contrast, flavosemiquinone species are observed to undergo rapid
dismutation in bulk solution.\textsuperscript{55} Thus, the appearance of a stable intermediate species
here suggests that the special environment of the ZMW nanopore facilitates the
stabilization of the flavosemiquinone species, behavior of radical redox species that is
not observed in bulk solution.

4.11 Reconciliation of ITO and ZMW Single-Molecule Spectroelectrochemical Data

As alluded to in Section 4.3.3.2, the stark differences between the bulk ITO and
confined ZMW single-molecule spectroelectrochemical data at each condition (\textit{vide supra}, Figure 4.16, Figure 4.17, Figure 4.18, and Figure 4.19) warrant further
consideration. Several phenomena contribute to these observations to varying degrees.

The first is that the relative effect of diffusion is vastly different between the two
experiments. Semi-infinite planar diffusion characterizes molecular transport in the bulk
solution above the ITO surface, which makes the process diffusion-limited. This causes a
time lag in the fluorescence response to the applied potential,\textsuperscript{97} as the majority of the
confocal probe volume samples the bulk solution beyond the diffusive boundary layer,
especially at the fastest CV scan rates. The more efficient hemispherical diffusion to the
mouth of the ZMW nanopores and the predicted thousands of wall collisions per second
inside of the pore result in the very rapid response of the fluorescence to the applied
potential.

The second is the effect of the local pH. The reduction of molecular oxygen is key
to understanding how the pH varies spatiotemporally. Three possible reduction half-
reactions through which oxygen is reduced are listed in order of decreasing thermodynamic favorability; the standard reduction potential, $E_0$, is shown relative to a standard hydrogen electrode (SHE),\textsuperscript{98}

\begin{align*}
O_2(g) + 4H^+ + 4e^- &\rightleftharpoons 2H_2O \quad (E^0 \approx +1.229 \text{ V vs. SHE}) \quad (4.2) \\
O_2(g) + 2H^+ + 2e^- &\rightleftharpoons 2H_2O_2 \quad (E^0 \approx +0.682 \text{ V vs. SHE}) \quad (4.3) \\
O_2(g) + 2H_2O + 4e^- &\rightleftharpoons 4OH^- (aq) \quad (E^0 \approx +0.401 \text{ V vs. SHE}) \quad (4.4)
\end{align*}

Across the range of applied potentials, -0.439 V to +0.061 V vs. Ag/AgCl (-0.203 V to +0.297 V vs. SHE), all three reaction pathways are accessible because $E_{appl}$ is always less than $E^0$. The availability of reactants will also contribute to which reactions proceed. Reactions 4.2 and 4.3 consume protons in solution, increasing the pH. At the same time, reaction 4.4 generates hydroxide ions, which also increase the pH. The primary result of an increase in pH is a shift in the $E_{1/2}$ value, which becomes more negative as the pH becomes more basic,\textsuperscript{65} as shown in Figure 4.1. For instance, a pH change from pH 2.90 to pH 4.80, shifts the $E_{1/2}$ value from -0.189 V to -0.300 V vs. Ag/AgCl (+0.047 V to -0.064 V vs. SHE). As such, only $E_{appl} < E_{1/2} = -0.300 \text{ V}$ would be reductive. Stated in another way, $E_{appl} = +0.061 \text{ V}$ is only +0.250 V greater than $E_{1/2}$ at pH 2.90, but +0.361 V greater than $E_{1/2}$ at pH 4.80. Correspondingly, the population of fully oxidized FMN and moderately fluorescent intermediate states would be greater than expected at pH 2.90.

In addition to its effect on local pH, molecular oxygen has two direct and opposed effects as it can either can quench fluorescence or oxidize reduced FMN molecules. Because the above reduction half-reactions consume molecular oxygen,
oxygen’s direct effect and the change in local pH are inversely related. At $E_{\text{appl}}$ values negative of $E_{1/2}$, the overpotential for Reactions 4.2-4.4 is the greatest. Conversely, the overpotential is the least for $E_{\text{appl}}$ values positive of $E_{1/2}$. Thus, dissolved O$_2$ has its greatest impact on the quenching of fluorescence when a large portion of the FMN population is oxidized. At all the $E_{\text{appl}}$ values above, the majority of molecular oxygen should be reduced near ITO surface and inside the entire ZMW nanopore. Thus, the observation volume of the ZMW is expected to have relatively few oxygen molecules and an increased pH. In contrast, the diffusive boundary layer only extends partially into the confocal probe volume in a bulk measurement, particularly with short potential steps and fast scan rates. This means that inside the confocal probe volume, there will be gradients of both oxygen concentration and pH. The oxygen concentration will decrease nearing the ITO electrode surface, while the pH will increase. At fast scan rates, the majority of the confocal probe volume will only be sampling the bulk solution beyond the diffusive boundary layer. Further experiments are needed to determine the relative rates of these processes.

Lastly, a mix of redox states is likely for a majority of the time, especially at $E_{\text{appl}}$ near $E_{1/2}$. This makes it difficult to precisely relate the observed fluorescence intensity to any one redox state of FMN. The distributions of these states will depend, in part, on the local pH. As stated above, the local pH profile can vary greatly between the confocal probe volume in bulk solution and the observation volume of a ZMW.

Other considerations deserve attention through simulations and experimentation. The above discussion does not incorporate the effect of buffer
capacity, via buffer concentration, on the mitigation of pH changes. Additionally, more basic pH values can greatly limit the population of fully protonated fully reduced species, simplifying the accessible redox pathways shown in Figure 1.1. Moreover, FMN’s lowest pKa value of 0.7, means that the phosphate group is deprotonated at least once, and correspondingly, has at least one negative charge at the pH values explored here.\textsuperscript{65} Since the majority of $E_{appl}$ values will leave the electrode surface with a density of negative charges, there will be some repulsion of the FMN species. Full ion-permselectivity in ZMWs is clearly not seen; otherwise, large changes in fluorescence intensity would not have been observed above. Section 5.3 details additional work that would provide a better understanding of the chemical and physical systems.

4.12 Concluding Remarks

The experiments reported here constitute the first single-molecule spectroelectrochemistry investigations in an electrochemical ZMW (E-ZMW) nanophotonic structure. The small observation volume (i.e., $V_{eff} \approx 200$ zL) of the conical Au-clad ZMWs made it possible to study the electroluminescence behavior of single flavin (FMN/FMNH\textsubscript{2}) molecules under both static and time-dependent potential control. Under static potential control, fluorescence intensity histograms are consistent with the expectation based on bulk studies that fluorescence increases as $E_{appl}$ becomes more positive. Interestingly, a non-zero fluorescence intensity is observed even at the most negative applied potentials, an observation that is attributed to the interplay
between the transport of oxidized and reduced molecules into and out of the ZMW nanopore and redox reactions at the Au working electrode.

Two types of time-dependent electroluminescence experiments were performed: chronofluorometry experiments, in which the potential is alternately stepped between oxidizing and reducing potentials, and fluorescence-reported cyclic potential sweep experiments, the fluorescence analog of cyclic voltammetry. Chronofluorometry measurements exhibit a fluorescence response strongly correlated to applied potential steps, but with an interesting asymmetry between the negative- and positive-going steps. The substantial fluorescence transient spike observed on the positive-going step is attributed to a large population of oxidized species that is depleted over time by diffusion of FMN out of the pore and photobleaching. Fluorescence-reported cyclic potential sweep experiments exhibit dramatic scan rate dependences with the slowest scan rates showing distinct intermediate states that are stable over a range of potentials. These states are tentatively assigned to flavosemiquinone species that are stabilized in the special environment of the ZMW nanopore.
CHAPTER 5:
FUTURE DIRECTIONS

5.1 Introduction

The instrumentation and experimental work described in preceding chapters provide the tools and framework for numerous studies, with both existing and additional capabilities. This chapter describes modular instrumentation upgrades or straightforward experimental modifications, which can be individually addressed in the laboratory, though the effect of addressing multiple issues is expected to be synergistic.

5.2 Instrumentation Optimization and Modification

The confocal fluorescence correlation spectroscopy optical microscope developed in Chapters 2 and 3 has been demonstrated to be capable of complex measurements. Several areas that are clear targets for future upgrades are to increase experimental yield, measurement accuracy, or expand the types of measurements that can be made. These will be described in the following sections.

5.2.1 Thermal Drift Mitigation

Issues associated with sample drift were detailed in Section 3.8. The impact of sample drift is determined by the smallest fundamental length in a given measurement.
It is unlikely that drift originates from a single source, but the one source that affects all optical measurements is thermal drift.\textsuperscript{99} Thermal drift is likely the principal causative factor in focus drift, which is a common issue in confocal imaging and is often addressed with hardware-based compensation or software-based correction.\textsuperscript{100-101} Thermal drift is the result of temperature changes and gradients acting upon the numerous solid materials used to construct the microscope, particularly aluminum and stainless steel. The linear expansion of a solid can be estimated as in Equation 5.1,

\[ \Delta l = \alpha l_0 \Delta T \]  \hspace{1cm} (5.1)

where \( \alpha \) is the material’s linear thermal expansion coefficient and \( l_0 \) is the material’s initial length.\textsuperscript{102} With a \( \alpha \) value of \( 17.3 \times 10^{-6} \text{ K}^{-1} \),\textsuperscript{102} a temperature change of +1 °C, would result in a 1.73 \( \mu \)m expansion of an initially 10 cm long piece of stainless steel. With a \( \alpha \) value of \( 23.1 \times 10^{-6} \text{ K}^{-1} \),\textsuperscript{102} a temperature change of +1 °C, would result in a 2.31 \( \mu \)m expansion of an initially 10 cm long piece of aluminum. Obviously, the different expansion rates applying to different materials make the problem more complex, but even if the construction material is the same throughout, a \( \approx 2 \) \( \mu \)m expansion can easily delocalize a 50 nm diameter nanopore and a 500 nm diameter confocal probe volume.

The most comprehensive approach to diminishing the effects of thermal drift is to enclose the entire instrument in an environmentally-controlled chamber. In addition to stabilizing temperature, such an enclosure would also provide humidity stabilization. Because the enclosure would need to be reasonably airtight, it would eliminate air currents around the sample. Because a vibration table serves as a large heatsink,
isolating the instrument from it is important. One possible implementation is to take the approach described in Section 2.3.2.4 by incorporating a raised breadboard. The breadboard would need to be large enough to support the entire instrument and would need to leave a gap of only a few inches between the table and the bottom of the breadboard. The instrument footprint could be reduced through a redesign to make the size of the necessary breadboard smaller. Pumping of cool or warm air could be done in the cavity below the breadboard, or through a large area diffuser in order to avoid introducing unwanted air currents. An automated feedback loop integrating thermocouples could provide temperature stability. Custom-commercial incubation chambers for the enclosure of microscope bodies for cell-based experiments are usually made of Plexiglas. The enclosure would need to be designed with removable walls to give easy access to the optics for adjustment. The light baffling detailed in Section 2.3.2.6 could be attached to the Plexiglas walls or built around it. Since the Plexiglas would provide the structural frame, an opaque tarp could be laid over the enclosure to serve the same purpose.

In various attempts to eliminate sample drift, an aluminum screw-driven translation stage and slide mount was designed, but not machined. It addresses potential mechanical drift through the use of high-tolerance rail-guides to stabilize the sample. A rendering of the design is shown in Figure 5.1.
Figure 5.1: Design of a screw-driven translation stage and slide mount. It would fit atop the xy piezoelectric flexure stage described in Section 3.4. Components are color-coded: frame (dark grey), x-axis translation frame (red), y-axis translation frame (orange), sample holder (light grey), and optical axis (blue).

5.2.2 Beam-Profiling

Alignment techniques were addressed in Section 2.4. As discussed in Section 2.4.1.3, intensity measurements are the primary means of determining the accuracy of the alignment. A clear drawback of this approach is that intensity measurements provide an indirect description of the beam’s properties, because it is integrating the beam’s intensity over an approximately 0.70 cm² area. A direct measurement would use
a laser beam profiler, such as Newport Corporation’s LBP2-HR-VIS model. It can characterize the cross-section beam width and shape, the effects of spherical aberrations, and other non-idealities with high-resolution 2-dimensional or 3-dimensional representations. As an add-on, Newport Corporation’s LBP2-SAM-BB beam sampling system increases the maximum incident power density to 50 W/cm² and restores the original beam polarization to the measurement. These two components together would enable the best possible alignment to be achieved.

5.2.3 Multiple Laser Integration with an Acousto-Optic Tunable Filter (AOTF)

A limitation of the current FCS optical microscope is its single monochromatic excitation source. A continuous-wave (cw) solid-state laser provides stable and reliable monochromatic excitation light, but with the trade-off that only chemical systems with strong absorption along the laser line can be investigated. Equipping the instrument with multiple excitation wavelengths can be done with a variety of strategies. While mercury and xenon lamps provide a broad spectrum of wavelengths to select from, even the most advanced excitation filters that have a full width at half maximum (FWHM) of 10-20 nm (ET405/10x, ET490/20x, ET555/20x, Chroma Technologies Corp., Bellows Falls, VT) fail to provide a fully monochromatic beam. A polychromatic beam is less favorable for confocal experiments than a monochromatic beam, because it produces a larger diffraction-limited confocal probe volume. A tunable solid-state laser such as the mode-locked Ti:sapphire laser (Tsunami, Spectra-Physics, Santa Clara, CA), available in the lab, enables selection of wavelengths over a wide spectral range, ca.
680-1,000 nm. Doubling and tripling crystals extend this range to ca. 230-500 nm, excluding a wide swath of the visible spectrum, ca. 500-680 nm. Unfortunately, the excluded range of wavelengths, 500-680 nm, includes the absorption spectra of many possible molecules of interest, an issue that is partially addressed in Section 5.4.

Integration of multiple continuous-wave (cw) solid-state lasers through acousto-optic tunable filter (AOTF) would address these issues. The 458 nm and 488 nm lasers that have been integrated into the microscope on separate occasions are part of Coherent Inc.’s Sapphire line of cw solid-state lasers. Two wavelengths within the same line that would provide access to a large portion of fluorophores in the visible range are 532 nm (Sapphire 532 LP) and 561 nm (Sapphire 561 LP). Integrating all four lasers into an AOTF would require combining them through the use of a combination of hot, dichroic, and cold mirrors. Dichroic and emission filter combinations would be required for each laser line. The ZT488rdc dichroic filter and ET525/50m emission filter from Chroma Technology Corporation could still be used for the 458 nm and 488 nm laser lines. For 532 nm, a ZT532rdc dichroic filter and an ET575/50m emission filter, both from Chroma Technology Corporation, could be used. Likewise, a ZT561rdc and an ET600/50m, also from Chroma Technology Corporation, could be used for 561 nm.

Figure 5.2 shows typical transmission spectra for each of these filter sets. A reliable means of switching between filter sets in the sample column would be required. A custom-mounted filter slider would serve this purpose (Zeiss Axioline 3FL Slider, Chroma Technology Corporation, Bellows Falls, VT).
Figure 5.2: Typical spectra data for three separate dichroic and emission filter sets, corresponding to laser lines of (a) 458 nm and 488 nm, (b) 532 nm, and (c) 561 nm.
5.2.4 Confocal Laser Scanning Microscopy (CLSM)

Currently, piezoelectric sample scanning, as discussed in Section 3.4.2, takes approximately 30-90 s, depending on the spatiotemporal resolution, to create a 100 μm x 100 μm image. Faster sample scanning is possible, but at the expense of introducing a non-negligible convective component into measurements of molecular diffusion. By keeping the sample stationary and scanning the laser, artifacts due to sample motion can be eliminated. Galvanometer mirror scanners for the x and y axes, along with support hardware and software, would have to be incorporated to scan the laser across the sample plane. Multiple raster scanned images could be obtained per second. In addition to faster scanning for image acquisition, it would give access to scanning fluorescence correlation spectroscopy (sFCS). Though not amenable to measurement of nanostructures, sFCS has been shown to provide more accurate measurement of diffusion coefficients than traditional FCS.93, 104

5.3 Experimental Modifications and Variations

As stated in Section 5.2, several instrumentation improvements, particularly thermal drift mitigation, should lead to greater experimental success rates. The investigation of several specific variables relating to the chemical sample and nanophotonic devices could provide a wealth of information about the transport phenomena and reactivity within the confined environment of ZMWs.
5.3.1 Experiments on Oxygen-Free Solutions

Analysis of spectroelectrochemical data discussed in Chapter 4 is complicated by the oxidation of reduced FMN molecules by molecular oxygen in solution. Because the molecules in a ZMW should be in the redox state corresponding to the applied potential, chemical oxidation while at a reductive potential is deleterious to the desired electrochemical measurements. By biasing the distribution of molecules towards oxidized or partially oxidized states, the overall background fluorescence signal is increased, thereby decreasing the difference in the fluorescence signal between oxidative and reductive applied potentials. Argon is a prime candidate for displacement of oxygen due to its greater mass and chemical inertness. A custom-sample holder would be needed to bubble Ar through solution prior to data acquisition.

5.3.2 Isolation of the Working Electrode (WE) within the Observation Volume

An interesting modification to the design of the ZMW arrays would be to limit the exposed working electrode (WE) area to the bottom of the nanopore. In the experiments described in Sections 4.8-4.10, the redox state of FMN molecules was actively manipulated along the entire exposed Au surface, including the bulk solution outside the pore and the pore volume that was not part of the observation volume. This means that most redox reactions occur outside the observation volume. That geometry makes it difficult to distinguish between fluorescence intensity spikes due to oxidized molecules entering the observation volume and reduced molecules being oxidized within the observation volume. A ZMW geometry that isolates the WE to the bottom of
the pore would isolate the source of fluorescence intensity increases to redox processes within the observation volume.

One approach would be to modify the devices made by Zaino et al., by depositing a 100 nm thick Au layer followed by a 100-200 nm thick SiN$_x$ dielectric layer on top. FIB milling through both layers would produce a nanopore with approximately the same overall physical dimensions, but with the WE surface exposed only at the 100 nm above the Au/glass interface. A potential drawback of this approach is that SiN$_x$ poorly attenuates the optical field. Thus, for nanopores with $d_{\text{bottom}}$ values greater than ca. 50 nm, a non-negligible contribution to the fluorescence signal may be made from the bulk solution.

An approach to isolate the WE to only the bottom surface of the pore would be to utilize the indium tin oxide (ITO) coated coverslips used in Section 4.3 as ready-to-use substrates. Rather than using Au-cladding, Al-cladding could be used as was done in the original demonstration of ZMWs, as in Section 4.4, to make the ITO surface the only working electrode surface. An aluminum oxide layer would form on the Al, passivating it. Using focused ion beam (FIB) milling as in Section 4.4 for the fabrication of ZMWs would be difficult because it would be hard to stop milling at the Al/ITO interface. Electron-beam lithography followed by reactive ion etching would provide adequate control to position the ITO electrode at the bottom of the nanopore.
5.3.3 Multi-Layer Zero-Mode Waveguide Devices

Ma et al. have shown the utility of recessed ring-disk nanoelectrode (RRDE) arrays for electrochemical detection and redox cycling.\textsuperscript{45, 105-107} Varying the geometry to have a transparent disk electrode, possibly ITO, or one that used a second ring electrode in place of the disk electrode, would enable spectroelectrochemical measurements to be made. The ring-disk ZMW (RD-ZMW) arrays would combine the current amplification of RRDEs with the high sensitivity of ZMW fluorescence measurements.

5.3.4 Residence Time Simulations

The initial Monte Carlo simulations of Brownian motion, discussed in Section 4.5, provided a reasonable first-order approximation of the mean residence time that a molecule spends inside the effective volume of a ZMW. Though straightforward to implement, the initial random starting position inside of the pore does not capture the complexity of molecules freely diffusing into, within, and out of the pore. A more complete simulation would be to start particles in random positions in the bulk solution above the physical pore volume. The residence time would be recorded as the time the particle spends inside the effective volume. Such a simulation would provide more accurate distributions of the residence time. Tracking individual particles could be used to determine the percentage of molecules that exit and reenter the effective volume, and the number of wall collisions before entering, and while in, the observation volume.
5.4 Other Fluorescent and Electroactive Molecules

Ackerman and coworkers highlighted the applicability of phenazines, phenoxazines, and phenothiazines cation dyes for electron transfer studies.⁵³⁻⁵⁴ Cresyl violet, a phenoxazine dye, has a quantum efficiency of ca. 0.54.⁹⁸ This makes it more attractive from a fluorescence standpoint than FMN with its ca. 0.3 quantum efficiency. Like FMN, it is strongly fluorescent in the oxidized state and weakly fluorescent in the reduced state. Cresyl violet absorbs strongly along the 561 nm laser line proposed in Section 5.2.3.ⁱ⁰⁹ The signal-to-background ratio observed in single-molecule spectroelectrochemistry measurements by Ackerman and coworkers was approximately 4-5,⁵³ which should be sufficient for reliable cross-correlation analysis.

5.5 Concluding Remarks

The work described in Chapters 2, 3, and 4, along with the future directions outlined here, contributes to the tools in the analytical toolbox that are a part of the grand challenge of the measurements made by lab-on-a-chip (LOC) analytical systems and point-of-care (POC) diagnostic devices. Direct spectroelectrochemical observations of single-molecule transport and reactivity in the confined geometry of ZMWs provide a framework for a variety of highly sensitive and selective detection schemes.
APPENDIX A:

BAFFLING SCHEMATICS

A.1 Description of Baffling Schematics

A discussion of the purpose of the baffling with a general description is found in Section 2.3.2.6. All of the baffling schematics are depicted in relation to threaded holes on the passively damped vibration table, which are alphanumerically labelled. Most baffling walls and support beams are assigned alphanumeric labels.

Figure A.1 shows a schematic of the exterior baffling walls with support braces and roof beams. The interior wall that separates the excitation and detection sides is also depicted. Figure A.2 highlights the interior baffling around detection pathways within the framework of the exterior baffling walls. Figure A.3 adds roof panels to the interior baffling highlighted in Figure A.2. Figure A.4 adds roof panels to the exterior baffling in Figure A.1.
Figure A.1: Configuration of exterior baffling and interior wall separating the excitation and detection sides in relation to threaded holes on the passively damped vibration table; support braces and roof beams are shown.
Figure A.2: Configuration of exterior baffling, interior wall separating the excitation and detection sides, and interior baffling around detection pathways in relation to threaded holes on the passively damped vibration table.
Figure A.3: Configuration of exterior baffling, interior wall separating the excitation and detection sides, and interior baffling around detection pathways in relation to threaded holes on the passively damped vibration table; roof panels over interior baffling are shown.
Figure A.4: Configuration of exterior baffling and interior wall separating the excitation and detection sides in relation to threaded holes on the passively damped vibration table; support braces, roof beams, and roof panels are shown.
   282 (5395), 1877-1882.
   36 (1), 151-169.
    fluorescence marker concentration as a probe of mobility. Biophys. J. 1976, 16 
    (11), 1315-1329.


95. Piruska, A.; Branagan, S.; Cropek, D. M.; Sweedler, J. V.; Bohn, P. W., Electrokinetically driven fluidic transport in integrated three-dimensional


