Fluorescence Spectroscopy for Cell Membrane Biophysics at the Nanometer and Nanosecond Scales

Christopher Randolph Rhodes

Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in Engineering – Mechanical Engineering

Designated Emphasis

in Nanoscale Science and Engineering

in the Graduate Division of the

University of California – Berkeley

Committee in charge:

Prof. Jay T. Groves, Co-Chair

Prof. Costas Grigoropoulos, Co-Chair

Prof. Samuel Mao

Prof. Daniel Fletcher

Fall 2012
Abstract

Fluorescence Spectroscopy for Cell Membrane Biophysics at the Nanometer and Nanosecond Scales

Christopher Rhodes

Doctor of Philosophy in Mechanical Engineering
Designated Emphasis in Nanoscale Science and Engineering

University of California – Berkeley

Prof. Jay T. Groves, Co-Chair
Prof. Costas Grigoropoulos, Co-Chair

Cell membranes organize and regulate a vital share of biological signaling. Fluorescence has become the main analytical tool with which to study such systems, and novel fluorescence techniques drive biological discovery at ever-smaller size and time scales. By exploiting the unique photophysics of fluorescent probes, I have advanced two parallel approaches to studying membrane systems: nanoengineered biointerfaces and time-resolved spectroscopy. Metallic nanostructures were engineered to precisely localize fluorescence emission, enhance label-free detection of lipid-protein interactions, and manipulate the diffusion landscapes of supported lipid bilayers. A platform for fluorescence spectroscopy at sub-nanosecond resolution combines fluorescence cross-correlation spectroscopy (FCCS) and time-resolved fluorescence anisotropy (TRFA) to characterize protein diffusion. These techniques have provided insight into the organization and structural flexibility of intercellular adhesion molecule-1 (ICAM-1), oligomerization of the small GTPase H-Ras, and the association of diverse lipid anchor domains in vitro and in vivo. Finally, I demonstrate progress toward single-molecule characterization of lipid-protein systems, and elaborate on its relevance to environmental biosensors for public health and biosecurity applications.
# TABLE OF CONTENTS

Table of contents .......................................................................................................................... i
Acknowledgments .......................................................................................................................... iii
1. Introduction ................................................................................................................................. 1
2. Nanostructures for fluorescence enhancement ......................................................................... 2
    Review of metal enhancement ..................................................................................................... 2
    Plasmon-enhanced immunostaining of interleukin secretion ..................................................... 3
    Finite-element simulations of near-field localization ................................................................. 10
    Conclusions ................................................................................................................................. 16
3. Nanometallic substrates for supported lipid bilayers ............................................................... 17
    Partitioned supported lipid bilayers ........................................................................................... 17
    Chromium grids – improvements in fabrication yield ................................................................. 19
    Major quality issues and corrective actions ............................................................................... 19
    Preferred fabrication protocol .................................................................................................. 24
    Protein patterning with sacrificial barium fluoride films ........................................................ 28
    Single-molecule Ras assays with zero-mode waveguides ......................................................... 30
    Conclusions ................................................................................................................................. 38
4. Time-resolved fluorescence at submicron resolution ............................................................... 39
    Introduction ................................................................................................................................. 39
    Fluorescence anisotropy and rotational correlation ................................................................. 39
    Viscous flow relations ............................................................................................................... 40
    Review of fluorescence anisotropy methods ............................................................................ 43
    Recovery of rotational correlation models from TRFA data .................................................... 44
    Hardware design ......................................................................................................................... 49
    Hardware setup protocol ............................................................................................................ 53
    Software for anisotropy fitting ................................................................................................... 58
    Correlation analysis for FCS and FCCS ..................................................................................... 62
    System characterization ............................................................................................................. 64
    Conclusions ................................................................................................................................. 66
5. TRFA of polyhistidine-attached proteins on bilayers ............................................................... 67
    Introduction ................................................................................................................................. 67
    Motivation .................................................................................................................................. 67
His10-GFP rotational diffusion: solution and membrane measurements .......................... 68
ICAM bilayers ............................................................................................................... 74
Anisotropy of ICAM-1 in T-cell immunological synapse ............................................. 75
Translational mobility and brightness in pSMAC ......................................................... 75
Discussion ...................................................................................................................... 76
Conclusions .................................................................................................................. 76
6. Fluorescence spectroscopy below the resolution limit .............................................. 78
   Introduction ................................................................................................................ 78
   Ras dimerization ....................................................................................................... 78
   Single-molecule photophysics in immobilized vesicles ............................................. 83
   Conclusions ................................................................................................................ 85
7. Future directions ....................................................................................................... 86
References ..................................................................................................................... 90
ACKNOWLEDGMENTS

Although I have never doubted that science is a team effort, I remain amazed at the number of collaborations that I have enjoyed since starting at UC Berkeley in August 2007.

I am indebted to Profs. Jay Groves (HHMI, LBNL, Chemistry) and Xiang Zhang (Mechanical Engineering) and their laboratories. Dr. Adam Smith (Univ. Akron), Dr. Wan-Chen Lin, and Dr. Christopher Gladden have provided immense support in thousands of ways, some reflected in author lists, others not. I also sincerely thank the NSF for the honor and immense opportunity presented by the Graduate Research Fellowship.

Prof. Theobald Lohmüller (TU-Munich), Dr. Hung-Jen Wu (Methodist Hospital Research Institute, Houston), and Dr. Sheng Wang have been major collaborators in nano-optics projects, for which I also acknowledge the contributions of Dr. Jason Valentine (Vanderbilt) and Dr. Tae-Jin Yim (PNNL). Prof. Michael Teitell, Prof. Yong Chen, Dr. Colin Stuart, and Dr. Sheraz Kalim were excellent collaborators and provided an overwhelmingly positive introduction to the community at UCLA.

I have had the pleasure of working with Dr. Lars Iversen (Univ. Copenhagen), Dr. Sune Christensen, Prof. Sara Triffo (Elon Univ.), Dr Niña Hartman (UCSD), Dr. Hector Huang, Hsiung-Lin Tu, and Eulanca Liu (UCSD) on a diverse range of fluorescence spectroscopy projects, and benefited from each researcher’s unique expertise, typically in the confines of a darkened, noisy microscope enclosure.

Dr. Sam Lord, Michael Coyle, and Geoff O’Donoghue have proven themselves to be outstanding resources in the Groves lab, and have frequently rescued experiments from last-minute complications. Hiroyuki Kai, William Huang, and Meredith Triplet will inherit the platforms developed in my dissertation research – teaching them has provided me with a much deeper understanding of these methods, and I have the greatest confidence in their visions for future research.

Dr. Rebecca Petit, and Xiaofan Meng and Jay Morford (Marvell Nanolab) have assisted with nanofabrication. More recently, I would like to thank Dr. Stephen Casalnuovo for helpful discussions of future research projects in environmental biosensing.

I am deeply honored to have worked alongside such an impressive body of experts. This multidisciplinary, collaborative spirit is without a doubt the basis of Berkeley’s important place in science.
1. INTRODUCTION

The frame of biological discovery has shifted during my lifetime, and in most ways is inextricably linked with the development of new engineering tools. Improvement of the optical microscope has historically driven biological discoveries at the level of organisms, tissues, and cells.

Photonic technology remains the primary medium of observation into biological interactions, but can now access a broad variety of physical variables that extend far beyond simple imaging. Fluorescent protein fusions avoid the need to exogenously stain samples and allow observation of molecular events in real time. Confocal and related imaging techniques can assemble three-dimensional models of cells. The diffraction limit to optical resolution can be circumvented using a number of scanning optical techniques with high localization precision. Beyond its function as a passive observer of biological events, photosensitive protein expression systems allow in vivo control of cell function.

However, today's dominant discoveries are at the molecular scale, and analytical tools must address the unique variables present in this drastically reduced environment. Nanometer-scale measurements present well-established challenges to resolution and contrast in microscope imaging. A more elusive aspect of specimen size, however, is that many biophysical interactions lack imaging contrast at any size scale. Even with infinite spatial resolution, for example, the motion of a single myosin molecule must be inferred from the signal dynamics of site-specific labels. Absent the ability to form images of molecules at this scale, light is increasingly used as a proxy signal for other physical variables such as chemical environment, molecular orientation, and protein conformation.

Random Brownian motion of biomolecules and their surrounding solvents is an integral part of their function at this scale. Translational and rotational diffusion can therefore be used as an indicator of the thermodynamics of a biological interaction. For example, the dimerization of two identical proteins is generally indistinguishable by the intensity of their fluorescence emission, but can be readily measured by their diffusivities. However, diffusion timescales vary from picoseconds to seconds, necessitating a large dynamic range in the time response of photonic methods.

Stochasticity is a crucial dimension of the natural world. Biology will never converge on a universal constant to describe, for example, the cellular copy number of a given protein in the way that physicists have deduced the speed of light. Indeed, the past decade has featured extensive investigation into the gene regulatory variations that contribute to such heterogeneity. The frame of heterogeneity analysis has simultaneously narrowed from whole organisms to single cells, and even the impact of single protein instances in a single cell. Consequently, engineering tools must be designed not just to converge on a single measurement result, but also to precisely describe the statistical distribution of this response, often across several orders of magnitude. This in turn necessitates the storage and analysis of large data sets, integrating computational approaches to statistical processing into the experimental process.

Size, diffusion, and stochasticity all present challenges to molecular-scale biological experiments, and invite major contributions from engineering researchers.
2. NANOSTRUCTURES FOR FLUORESCENCE ENHANCEMENT

Review of metal enhancement

Diverse probe molecules are employed to study biological interactions. Fluorescent and scattering probes both produce photonic signals from the emission of excited electrical dipoles\textsuperscript{11}. The dependence of such emission on environmental factors has long been understood from electromagnetic theory\textsuperscript{12}, and metallic nanostructures have been applied to exploit this rate effect in many spectroscopy methods. The highest control over enhancement is typically achieved near nanostructured metallic surfaces that exhibit localized surface plasmonic resonance (LSPR), itself a commonly used technique to monitor adsorption of mass to a sensor surface\textsuperscript{13,14}.

The most striking quantitative enhancements have been achieved using surface-enhanced Raman scattering (SERS), whereby a multitude of metal surface effects can enhance emission intensity by a factor of 10\textsuperscript{14} or more. Early attempts to enhance pyridine infrared spectroscopy serendipitously revealed electromagnetic contributions to Raman scattering on rough silver electrode surfaces\textsuperscript{15–17}. Since then, enhancement factors have improved steadily in both resonant and non-resonant nanostructures\textsuperscript{18}. SERS is now a widely used technique in a variety of nanoparticle geometries\textsuperscript{19,20}, including assays with single-molecule sensitivity\textsuperscript{21}.

Considering that similar SERS effects have been observed on randomly adsorbed metal colloid films as carefully engineered nanostructures, surface roughness is a crucial variable in light-metal interactions\textsuperscript{22}. Large-area metal films are known to enhance Raman and fluorescence in unidentifiably random “hot spots”. These have recently been measured at sub-100 nm dimensions using near-field imaging of randomly diffusing nanoparticles, although electromagnetic field distribution profiles were not related to any identifiable surface topography features\textsuperscript{23}. Perhaps the most striking evidence that surface roughness directly influences near-field spectroscopy enhancements was provided by comparisons on metal films prepared using an ultra-smooth template-stripping protocol\textsuperscript{24}. Comparison of conventionally prepared and template-stripped films showed that roughness features directly cause hot spots, and that reduced SERS signal can be rescued on smooth films by adding an additional rough film\textsuperscript{25}. The same fabrication technique also improved plasmonic waveguide propagation efficiency by decreasing scattering losses at the film boundary\textsuperscript{26}.

Fluorescent labeling is a fundamental tool in molecular biology and biophysics, and plasmonic interactions have been similarly explored to enhance fluorescence\textsuperscript{1,27}. Proximity to a metallic surface can alter both radiative and nonradiative decay rates of a fluorophore, and extensive theoretical calculations predict that dipole radiation should show an oscillatory dependence on distance from a planar metallic surface\textsuperscript{28} or metal nanoparticles\textsuperscript{29}. This effect can therefore selectively enhance or quench fluorescence, and has been verified on single molecules immobilized in thin films\textsuperscript{30}. Other studies have validated this using DNA spacers to precisely control the distance between fluorophores. Fluorescence lifetime measurements demonstrated that an increase in fluorophore nonradiative decay rate is responsible for quenching effects in distance ranges from 2-16 nm\textsuperscript{31–33}.
In addition to enhancing the rate of light emission, nanoscale effects can be exploited to localize optical excitation at size scales far below the diffraction limit. This nanofocusing effect has been achieved in biological systems using near-field optical scanning microscopy (NSOM), although the cumbersome instrumentation limits this technique’s usefulness for high-yield assays. Sophisticated nanostructures such as multilayer hyperlenses and corrugated waveplates can also achieve subwavelength focusing, but with similar limitations. Because statistically significant characterizations of many biological interactions require either solubilized or large-area ensembles of measurements, spectroscopic methods that exploit the near-field interactions of simple metallic nanoparticles are attractive.

Plasmon-enhanced immunostaining of interleukin secretion

Overview

Nanoimprint lithography was used to pattern large-area arrays of plasmonic nanoresonators. This platform enhanced fluorescent immunostaining of interleukin-2 (IL-2) secreted from T-cells, as reported in “Subcellular Resolution Mapping of Endogenous Cytokine Secretion by Nano-Plasmonic-Resonator Sensor Array”.

Figure 1 – (A) Tunable nanoplasmnic resonator (TNPR) cylindrical cross-section. Local electric field is enhanced around perimeter of Au-SiO$_2$ interface. (B) Functionalization and assay schematic for enhanced immunostaining of interleukin-2 (IL-2) captured on TNPR surface with primary antibody. (C) Schematic for large-area secretion mapping. (D) Schematic for intracellular measurements with solubilized TNPRs.
Introduction

Plasmonic nanostructures typically exhibit resonances that derive from both the material’s composition and the structure’s geometry. In order to utilize the latter effect for a variety of Raman and fluorescence probes, tunable nanoplasmonic resonators (TNPRs) were developed to tune infrared resonance using an internal silicon dioxide (SiO$_2$) film of variable thickness between two gold layers (Figure 1A)\textsuperscript{40,41}. TNPRs were fabricated using electron beam lithography (EBL) to pattern polymethylmethacrylate (PMMA) resist film, and the Au/SiO$_2$/Au layers deposited using electron-beam and thermal evaporation. Solvent treatment then lifted off the Au/SiO$_2$/Au film stack from unexposed resist (Figure 2A), leaving only cylindrical TNPRs on the substrate. Earlier quantification of enzyme activity using TNPR-enhanced SERS detection showed promising applications to a variety of \textit{in situ} biochemical characterization assays\textsuperscript{42}. Extension of this platform to fluorescence immunoassays, in which analyte molecules were specifically captured and identified by antibodies, was a natural next step in this project (Figure 1B).

Although it offered precise dimensional control, the long exposure times needed for EBL limited the size of TNPR arrays to the thousands. Large-area fabrication was desired for two main applications. First, TNPR-coated surfaces could be used to enhance the intensity of diverse \textit{in vivo} signals by randomly depositing cells on enhancement arrays, a platform that could measure only a small number of cells with the limited surface areas available from EBL lithography (Figure 1C). Second, large arrays could potentially be solubilized and concentrated, enabling plasmon-enhanced measurements of analyte binding in solution (Figure 1D). By solubilizing large-area lithographically patterned TNPR arrays, sufficient TNPR concentrations could conceivably be achieved for ballistic injection into cells, providing \textit{in vivo} characterization of protein expression.

Figure 2 – (A) Top-down lithography process of gold TNPRs using either electron beam or UV laser interference exposure; (B) Two-layer nanoimprint lithography implemented for large-scale fabrication.
Large-scale fabrication: laser interference lithography

Initial attempts at large-scale TNPR fabrication utilized laser-interference lithography (LIL) to pattern holes in UV-sensitive photoresist. Silicon wafers were coated with XHRIC-16 antireflection coating (Brewer Scientific, Rolla, MO) and NR7-250P photoresist (Futurrex, Franklin, NJ). A 364 nm argon laser beam was then expanded and split by a mirror to form an interference pattern (Figure 3A). Samples were exposed once and then a second time after rotating 90°, resulting in a symmetric grid of round holes after treatment in diluted Futurrex RD6 developer. Film evaporation then followed a similar procedure to that for EBL exposure (Figure 2A).

Following evaporation of the Au/SiO$_2$/Au stack, liftoff in acetone removed very little of the film even after aggressive sonication for 10 minutes or more. Detailed inspection in SEM revealed few instances of successfully patterned circular structures. To troubleshoot this process, a single LIL exposure was performed, and the silicon substrate cleaved along a perpendicular plain to image the resist profile in SEM cross section. This revealed a roughly sinusoidal modulation of film thickness (Figure 3B–D), suggesting that resist sidewall profiles were not sufficiently sharp for film liftoff to occur. By projecting UV interference fringes onto a paper screen, problems with contrast and stability of the exposure pattern were identified. Modifications to the optical system, particularly construction of a plastic enclosure to reduce thermal fluctuations, substantially improved
LIL fabrication. Nonetheless, yields were too low to achieve the desired fabrication of large-area TNPR arrays.

_Nanoimprint lithography_

Considering these problems, an alternative fabrication strategy using nanoimprint lithography (NIL) was developed. NIL patterning for liftoff followed a two-layer protocol developed and executed in the laboratory of Prof. Yong Chen at UCLA (Figure 2B). To allow UV transmission during imprint and exposure, glass substrates were substituted for silicon. Prior to imprinting, a 100 nm film of PMMA was spin coated on the substrate, soft baked, and its thickness confirmed on a NanoSpec optical film measurement system (Nanometrics, Milpitas, CA). A second film of a proprietary UV-sensitive imprint polymer was then coated and remained in its liquid state: because of the volatility of its solvent, time between spin coating and imprinting was minimized. Holes were then vacuum sealed, imprinted from a silicon master, and exposed to UV illumination on a Karl Süss aligner (Süss Microtech, Garching, Germany) with a modified vacuum fixture.

A resist profile suitable for high-yield liftoff was obtained by oxygen plasma etching. The lower PMMA film etched at a substantially faster rate than the upper UV-sensitive resist layer, resulting in an undercut profile. The process was first carried out with an etching system in the nanofabrication cleanroom at the UCLA Nanoelectronics Research Facility, and later at the UC Berkeley Biomolecular Nanotechnology Center (BNC).

The desired undercut resist profile was confirmed by the presence of a clear shadow in NIL-patterned holes in SEM (Figure 4A-B). Film deposition was then carried out on a Torr electron beam evaporator (Torr International, New Windsor, NY). Because gold adhesion to glass is known to be poor, a thin 3-5 nm chromium adhesion layer was first deposited, then the same TNPR Au/SiO$_2$/Au stack described earlier. Fabrication of uniform TNPR arrays across arrays of 1 cm$^2$ or more was confirmed in SEM (Figure 4C-D).
TNPR solubilization

Solubilization of TNPRs from substrates into solution was explored using a number of strategies. In the case of LIL fabrication, the underlying antireflection coating was etched in an oxygen plasma. This redistributed TNPRs on the surface, although solubilization was never demonstrated. Because of the poor yield of LIL, however, this approach was not pursued further.

Sacrificial films of chromium (Cr) and barium fluoride (BaF₂) were separately used in sonication baths. Cr films were briefly etched using CR-7 chromium etchant and then solvent exchanged with methanol in a centrifuge vial. When redeposited on silicon and imaged in SEM, however, it became apparent that the aggressive etchant drastically reduced the size of TNPRs below 100 nm and disintegrated the constituent layers (Figure 5A-B). Compared to chromium, BaF₂ etches readily water, and yielded a small number of TNPRs that were redeposited on copper grids for analysis in a transmission electron microscope (TEM). TEM images showed several instances in which pairs of conductive films were separated by a nonconductive layer, all at the predicted dimensions for the nominal TNPR geometry (Figure 5C). A significant draft angle was also observed, possibly...
Figure 5 – (A-B) TNPRs solubilized and resorbed using wet etching of chromium adhesion layer. Small particle size was likely due to disintegration at gold-silicon dioxide interface, as well as chromium etchant attacking gold. (C) Low-yield direct solubilization of TNPRs in TEM, showing clear bevel on top gold layer.

A feature of the film deposition profile itself. Many instances of disintegrated conductive particles were also observed in TEM, suggesting that sonication was disrupting Au-SiO$_2$ adhesion.

**Functionalization with primary antibody**

Enhancement of fluorescence assays relied on primary antibody to capture analyte molecules at the surface of TNPRs (Figure 1B). Specific antibody adsorption to gold films was desirable for high image contrast and to reduce depletion of analyte molecules from unenhanced parts of the surface. For simplicity, functionalization was optimized on arrays of gold nanoparticles with similar dimensions to TNRPs. Nonspecific adsorption of labeled antibody (anti-IgG-Texas Red) was mitigated by etching the substrate for two minutes in oxygen plasma, resulting in high specificity to gold particles (Figure 6A), which may have themselves nominally enhanced the intensity of Texas Red emission. A similar functionalization protocol was then extended to IL-2 sensing experiments.
**Interleukin secretion assay**

Substrate-immobilized TNPR arrays were used to enhance staining of interleukin-2 (IL-2) secretion by Jurkat T-cells in the sandwich configuration pictured in Figure 1B. First, standard calibration curves were measured against purified IL-2 of known concentration, demonstrating somewhat heterogeneous responses across the image plane (Figure 6B) at high concentration to 100 μg/mL. This data was then used to calibrate image intensity against effective IL-2 solution concentration (Figure 6C), as well as signal-to-noise ratio (SNR) based on the statistical variance in each calibration image. Following these measurements, Jurkat T-cells were introduced to TNPR arrays and IL-2 secretion induced (Figure 6D). Secretion patterns appear along distinct radial gradients that were predicted from studies of interleukin autoinhibition. However, contact surfaces themselves did not show IL-2, indicating that perfusion is severely limited in this contact region. Enhanced IL-2 detection was not visible in substrate areas that were not coated in TNPRs, confirming that these structures enhanced fluorescent staining.

**Conclusions**

TNPR arrays modestly enhanced immunostaining of secreted IL-2 by an approximate factor of ten compared to adsorption on bare glass. Although plasmonic effects were the intended mechanism of this enhancement, high adsorption yield of primary antibody to
gold surfaces in TNPRs may have also played a role. A key challenge with this sensing platform is adsorption kinetics, both of the analyte itself and the secondary staining antibody. This assay lasted several hours, and hence the platform was not readily capable of monitoring real-time regulation or rapid secretion responses to stimuli. Nonetheless, this represents an important step in the adoption of nano-optical technology for sensing biological molecules, and serves as a starting point for further development of sensing techniques that specifically address secretion of small molecules such as interleukins.

**Finite-element simulations of near-field localization**

**FEA simulations overview**

Optical near-fields at plasmonic resonance can only be analytically calculated in very simple geometries. In most real nanoparticles, numerical simulation of Maxwell’s equations can be used to predict emission spectra and determine the localization of enhanced electric fields. Although finite-difference time-domain (FDTD) simulations are less computationally demanding, finite-element analysis (FEA) is readily feasible with modern computers and offers more accurate results near nanostructure boundaries. Because FEA directly solves the electric field, mean mesh element size must be several times smaller than the wavelength of the simulation. Although calculation of near-field nano-optical effects is reasonable with current computational hardware, memory and computation time demands makes such an approach unfeasible for far-field effects that require larger-volume models. The simulation geometries presented here were defined with limited volumes to model near-field effects and interaction spectra.

**COMSOL principles and settings**

COMSOL, formerly FEMLAB, is a platform for finite element analysis that can solve a diversity of physical models. COMSOL has become the standard for optics simulations in the nano-optics literature. A major advantage of COMSOL, although not specifically used in this work, is its ability to couple solutions of numerous physical effects, such as optics and heating, in a single simulation. Other attractive features of COMSOL are the ability to batch-process numerous simulations that vary frequency or other arbitrary simulation parameters. Simulations described here were performed using the RF Toolbox, wherein Maxwell’s equations are solved for given boundary conditions.

Because these particles were used in the context of fluorescence and scattering experiments, only scattered electromagnetic fields were calculated. Using these features, field and spectra were computed for a background field of arbitrary 1 V/m amplitude along the polarization vector of interest. Geometries were simulated in frequency-dependent mode, in which a frequency-independent kernel solution is first calculated. This specific case of frequency sweeping does not recalculate the entire solution for each frequency point, allowing absorption and scattering spectra to be computed at high wavelength resolution with a modest increase in computation time. Other parameter sweeps, including such parameters as medium index of refraction and model dimensions, require recalculation of the entire simulation, although this can be executed in batch mode for minimal user intervention.
The main output of each simulation was a full three-dimensional description of the electric field. The parameters of interest in the following studies were: 1) Localization of enhanced electromagnetic field relative to the particle geometry, and 2) particle scattering and absorption spectra. Local $E$-field enhancement was typically plotted as $\text{norm}(E)$:

$$\text{norm}(E) = \sqrt{E_x^2 + E_y^2 + E_z^2}$$  \hspace{1cm} \text{Eq. 1}

for the $x$, $y$, and $z$ components of the scattered electrical field. FEA mesh statistics are described in detail for silver nanocube and gold nano-antenna studies. To demonstrate that mesh densities were suitably high, single parameter conditions were simulated at meshes refined to about twice the base element density and convergence of key $\text{norm}(E)$ values confirmed.

Modeling material dispersion, the frequency-dependent variation in real and imaginary components of refractive index, is crucial for LSPR simulations. Silver (Figure 7A) and gold (Figure 7B) models were adapted from published dispersion tables. As noted in Figure 7B, gold exhibits an intersubband resonance near 600 nm that result in singularities in both real and imaginary refractive indices. This effect was not included in these FEA models, although it may account for a disproportionate increase in local enhancement in that narrow excitation band.

**Silver nanocubes**

FEA was used to model the LSPR distribution of silver nanocubes coated with silicon dioxide shells, including the penetration of enhanced electric field away from the silver surface into analyte solution. This study was included in “Membrane-protein binding measured with solution-phase plasmonic nanocube sensors”, currently in press for publication in Nature Methods.
Even with properly defined boundary conditions, reflection artifacts are known to appear in nanoscale EM simulations. The silver nanocube model was therefore surrounded by perfectly-matched layers (PML), volume features in COMSOL that, when defined to surround the features of interest, serve as empty volumes that extend to infinity. This simulates particle scattering in an ideally dilute environment and eliminates back-reflections from simulation results.

The nanocube geometry was adapted from a TEM image that described the nominal length dimension, SiO$_2$ shell thickness, and corner curvature radius. A free tetrahedral mesh of 21 nm maximum element size was generated throughout the 3d model, and further refined to 12 nm in and near the SiO$_2$ shell, for a total of 355,000 elements. Shell SiO$_2$ refractive index was fixed at 1.42, and solution index swept for five different values. Models were solved for scattered electric field with oscillatory background field of arbitrary 1 V/m.

Absorption spectra were computed in post-processing by integrating resistive power loss $Q_r$ over the model’s volume:

Figure 8 – FEA of silver nanocube resonance. Absorption (A) and scattering (B) spectra dependence on medium index $n$. Enhancement of local electrical field (C) is highest at rounded corners of nanocube at quadrupole peak and (D) penetrates through silica layer into medium.
\[
abs = \iiint_Q(r) \, dV \quad \text{Eq. 2}
\]

As expected, peak absorption occurs around 490 nm at \( n = 1.33 \) and gradually red shifts with refractive index of the medium (Figure 8A). Scattering spectra were similarly computed by surface integration of the electric field norm at the boundary between medium and PML:

\[
sca = \iint_s \| \vec{E} \| \, dA \quad \text{Eq. 3}
\]

Scattering spectra (Figure 8B) exhibited distinct dipole and quadrupole peaks around 500 nm and 650 nm, respectively, and were confirmed by the symmetry of enhanced electric field at the particle’s exterior surface (Figure 8C).

Of particular interest to this study was the putative shielding effect of the SiO\(_2\) shell on the particle’s LSPR sensitivity. A diagonal cross section through the nanocube’s corner (Figure 8D) showed that field attenuation does indeed occur within the shell, but the near-field is still significantly enhanced at the shell-medium interface. This proves that this silver nanocube assay maintains sensitivity to mass adsorbed on this SiO\(_2\) passivation shell, and therefore allows solution-phase characterization of binding events at this surface.
Gold nanoantennas

Nano-optical confinement in gold nanoantenna gaps was modeled using COMSOL. These results are included in “Single Molecule Tracking on Supported Membranes with Arrays of Optical Nanoantennas”, Nano Letters Vol. 12 (2012) pp. 1717-1721.

Extraordinary fluorescence intensity enhancements and field localization of 10 nm or smaller have been reported in gaps between triangular dimer nanoantennas. Enhancements as high as 1340 were previously achieved using a near-infrared dye in polymethylmethacrylate (PMMA) films prepared from toluene solution. Although these solvent conditions would preclude incorporation of this exact system in biologically relevant measurements, this nonetheless suggested that bowtie nanoarrays were a powerful tool with which to observe very small optical volumes in useful biological assays.

Previous bowtie arrays had been manufactured using electron-beam lithography. Large-area fabrication is essential for statistically significant biological experiments, and nanobowtie hexagonal arrays at areas on the order of 1 cm² were fabricated using self-
assembly of polystyrene particles. By engineering a subsequent reactive ion etching process, small polymer bridges were formed between adjacent particles. This then served as a mask for gold deposition using thermal evaporation, in which the directionality of the evaporation allowed the bridge to pattern a controllable gap between adjacent triangular particles.

Nano-antenna geometries were interpreted from high-resolution SEM images that described length dimensions and tip curvature radius (Figure 9A-D) and reconstructed in AutoCAD (Autodesk, San Rafael, CA). Furthermore, the edge of the gold film was expected to be rounded, and was modeled as an external fillet with 12 nm radius based on the gradient of intensity in SEM. Gold features were model on top of a cylindrical glass substrate of refractive index $n = 1.49$ (Figure 9B), and free scattering boundary conditions applied to all external surfaces. A free tetrahedral mesh with 223,000 elements was generated in COMSOL with 70 nm maximum element size, but was automatically refined near smaller features (Figure 10A).

The scattered field was solved from with varying frequency corresponding to 400-1500 nm excitation wavelength. Electric field was enhanced by a factor of 40 or more, corresponding to local intensity enhancements of at least 500 in a majority of the 10 nm
gap (Figure 10B-C). Dipole resonance near 700 nm was substantially stronger with a 10 nm gap than 127 nm, but did not shift appreciably (Figure 10D). Resonances further into the infrared increased accordingly.

These results confirm that the fluorescence excitation field is strongly enhanced in the gap between gold particle features, and verify the localization of nanofocusing used in supported lipid bilayer tracking experiments. In theory, the hexagonal symmetry of these structures will alter their spectral response. The main parameter of interest in these simulations was the location of highly enhanced fluorescence intensity between antenna tips, however, so only a single dimer was simulated. Furthermore, the gap between antennas is small compared to the surface extent of the gold film, so plasmonic interactions in the gap were expected to be fairly decoupled from symmetry effects. However, this parameter can be easily explored from the existing models by changing the boundary conditions for triangular symmetry (Figure 9D).

**Conclusions**

Although plasmonic effects in TNPRs enhanced fluorescence emission in IL-2 immunostaining experiments, the kinetics of antibody binding seriously limit the usefulness of this platform for *in situ* monitoring of biological signals. Theory predicts that surface effects can both enhance and suppress fluorescence, and indeed this effect was explicitly characterized in FEA simulations that accounted for silver nanocube oxidation layers. Nonetheless, the sensitivity of biosensing techniques based on plasmonic effects would strongly benefit from more specific characterization of distance-dependent rate effects, as the thickness of functionalization layers themselves may actually adversely affect enhancement and localization.

Perhaps a greater strength of plasmonic enhancement is the ability to focus light at a small area on metallic surfaces. This is particularly attractive for membrane-functionalized nanoparticle sensors that provide a well-mixed reagent pool, and can possibly improve single-molecule photonic measurements despite high analyte concentrations. As described in the following chapter, highly fluid supported lipid bilayers can be manipulated using nanofabricated structures, and this approach can address diffusion in nano-optical biosensors.
3. NANOMETALLIC SUBSTRATES FOR SUPPORTED LIPID BILAYERS

Partitioned supported lipid bilayers

Self-assembled, fluid supported lipid bilayers (SLBs) are a versatile model of cell membranes, and have featured prominently in many biophysical interrogations of membrane signaling systems\textsuperscript{51-53}. The extension of nanofabrication methods to biological interfaces has similarly provided extraordinarily precise control over surface chemistry\textsuperscript{54}, with many applications in basic bioscience experiments.

A diversity of strategies have been reported to pattern lipid bilayer surfaces\textsuperscript{55}. SLBs were partitioned into separate corrals by simply scratching the substrate surface, inhibiting the fluidity across the scratched area\textsuperscript{56,57}. Reducing solution pH then caused lipid bilayers to overwrite these scratched boundaries\textsuperscript{58}. To achieve more precise and smaller-scale manipulation of bilayer fluidity, however, a simple and versatile strategy based on nanofabrication has been applied to a broad variety of SLB applications. Prior to SLB formation, oxidized silicon or fused silica substrates were formed by depositing a thin film through a lithographically defined resist mask. Aluminum oxide lines defined by UV photolithography defined distinct corrals of supported lipid bilayers in which electrophoretic gradients could be imposed\textsuperscript{59,60}. Further study of this effect concluded that aluminum oxide actually prevents SUV fusion, hence inhibiting lateral diffusion across the corral.

Similar partitioning results were achieved with chromium, gold, and indium tin oxide (ITO), although observations of fluorescent lipids through grids suggested that direct immobilization of lipid molecules, rather than inhibition of vesicle fusion, accounted for this system’s partitioning effect\textsuperscript{61}. In particular, chromium thin film adhesion to glass is exceptionally strong compared to other commonly evaporated films such as aluminum and copper\textsuperscript{43}. Interaction of thin film metal oxide is known to improve adhesion to glass, as does substrate cleanliness\textsuperscript{62}. For this reason, chromium has proven to be the most durable thin film for this application.

This chromium protocol has been used to interfere with spatial organization of membrane receptors in numerous membrane signaling systems\textsuperscript{63-65}. More recently, the characteristic lateral fluidity of SLBs has also been used to maintain enzymatic access to single DNA strands that are tethered in the plane of the substrate via immobilized neutravidin\textsuperscript{66}. This strategy has been used to achieve single-molecule directional tethering via chromium\textsuperscript{67-70} and hydrogen silsesquioxane (HSQ)\textsuperscript{71} nanopatterns.

Considering the ongoing utility of SLBs partitioned by nanofabricated metal films, reproducibility and yield are crucial considerations for this platform’s continued growth to large-area measurements of cell signaling. Analogous interventions that rely on nanofabrication to engineer the mobility of reconstituted membranes offer additional opportunities to precisely characterize related biological functions.
Table 1: Chromium grid fabrication protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Material</th>
<th>Qty</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Starting material</td>
<td>Circular coverglass, e.g. Fisherbrand #1.5</td>
<td>1</td>
<td>ea</td>
</tr>
<tr>
<td>1</td>
<td>Soak substrates minimum 30 minutes in 1.25% Hellmanex II</td>
<td>Hellmanex II</td>
<td>1</td>
<td>mL</td>
</tr>
<tr>
<td></td>
<td>(Helma GmbH, Muellheim, Germany)</td>
<td>Deionized water</td>
<td>80</td>
<td>mL</td>
</tr>
<tr>
<td>2</td>
<td>Piranha clean minimum 5 minutes</td>
<td>Deionized water</td>
<td>100</td>
<td>mL</td>
</tr>
</tbody>
</table>

Warning: work with piranha only in dedicated hood and in accordance with SOP

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Material</th>
<th>Qty</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Dip coverglass five times in deionized water, refilling water beaker each time</td>
<td>Deionized water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Rinse both sides with isopropanol squirt bottle</td>
<td>Isopropanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Dry with compressed nitrogen then keep substrate covered</td>
<td>Compressed nitrogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Deposit dilute Zeon Chemicals ZEP-520A electron beam resist through PTFE syringe filter</td>
<td>ZEP-520A</td>
<td>0.5</td>
<td>mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Corning 0.45 μm PTFE syringe filter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Spin coat ZEP-520 40 sec. at 1000 rpm, ramp up 500 rpm/s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Bake 5 min. on hotplate at 145 degC setpoint, cool at least 2 min. on aluminum foil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Deposit aquaSAVE conductive polymer</td>
<td>Mitsubishi Rayon aquaSAVE</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: conductive polymer does not wet well to resist, so ensure that substrate area to be written is fully covered; conductive polymer is very difficult to procure.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Material</th>
<th>Qty</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Spin coat polymer 40 sec. at 1000 rpm, ramp up 500 rpm/s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Bake 5 min. on hotplate at 145 degC setpoint, cool at least 2 min.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Electron beam lithography: 100 pA, 1.0 us shot time on Crestec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Rinse conductive polymer in water, dry immediately in nitrogen</td>
<td>Deionized water</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: retain rinse residue as hazardous chemical waste

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Material</th>
<th>Qty</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Swirl 60 sec. in isoamyl acetate bath (synonym: isopentyl acetate)</td>
<td>Isoamyl acetate</td>
<td>100</td>
<td>mL</td>
</tr>
</tbody>
</table>

Note: dispose of isoamyl acetate as organic waste

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Material</th>
<th>Qty</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Rinse substrate in isopropanol, dry with compressed nitrogen, store covered</td>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isopropanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compressed nitrogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Electron beam evaporation: 5nm chromium on edwardseb3, nominal 31 mA beam current</td>
<td>Dichloromethane</td>
<td>100</td>
<td>mL</td>
</tr>
<tr>
<td>17</td>
<td>Lift off resist by dipping in dichloromethane, transferring to second dichloromethane bath, and sonicating 10 min.</td>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isopropanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compressed nitrogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Rinse substrate in water and isopropanol, dry with compressed nitrogen</td>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isopropanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compressed nitrogen</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chromium grids were fabricated using electron beam lithography (EBL) on a Crestec CABL-9510CC (Crestec Corp., Tokyo, Japan). This defines features for subsequent physical vapor deposition (PVD) of chromium films that are nominally 5 nm thick (Figure 11) in an Edwards EB3 electron beam evaporator (Crawley, UK). Chromium atop unexposed resist was then removed in a “liftoff” step by soaking in dichloromethane. Although mass-produced substrates have been explored using nanoimprint lithography, this process remains the most reliable and flexible means of producing grids. Two major classes of quality problems were addressed, leading to specific changes to a preferred fabrication protocol (Table 1).

Major quality issues and corrective actions

Residual metal and readsorption

During the liftoff portion of this protocol, substrates were first soaked for less than five seconds in a dichloromethane bath, then transferred to a second bath for about 10 minutes. However, square patches of residual chromium often compromise the usefulness of entire grid fields, and were readily observable in brightfield microscopy at low magnification. Total surface coverage of this effect varied widely between substrates in a batch as well as on a single substrate (Figure 12). This problem is consistent with incomplete dissolution of the underlying resist during liftoff in dichloromethane, so prolonging the second dichloromethane soak was explored. Despite drastically increasing the substrate’s time in the bath, as well as sonicating aggressively, no improvement was observed in patterns with a high prevalence of residual chromium (Figure 13).
Figure 12 – Surface coverage of residual chromium varied in severity between samples in a batch (A-D) and even between similar fields on the same sample (C-D). All images are 4 µm grids under 10x brightfield illumination.

Figure 13 – Additional liftoff sonication does not improve residual or readsorbed chromium. Same 4 µm grid after 10 minutes (A) and 45 minutes (B) of sonication in dichloromethane, imaged in 10x brightfield microscope.
Figure 14 – Subtle readsorption of chromium film frequently manifests itself as outright liftoff failure. (A) displaced individual squares are clearly observable in FEI Quanta SEM of 2 µm grids, (B) higher magnification reveals square displacements of less than 100 nm, essentially readsorbing chromium in the same location during liftoff.

Figure 15 – Several additional instances of residual chromium form images (A, B) that coincide with 600 µm EBL fields (C).
To further investigate the root cause of this problem, scanning electron microscopy (SEM) was used to image residual regions of chromium after liftoff. Surprisingly, many such squares showed slight displacements in high resolution images that were not observable in the light microscope (Figure 14). This strongly suggested that initial liftoff was indeed occurring as designed, but many chromium squares were then rapidly readorsorbed rather than being removed into the solution bath. Following this conclusion, the liftoff protocol was modified, and both dichloromethane baths were placed in a sonicator. The substrate was rapidly submerged in the first bath and immediately transferred to the second. This appeared to rapidly disintegrate chromium during liftoff, and substantially reduced the prevalence of residual chromium squares in this process.

Despite these improvements, this problem still appeared sporadically. In several cases, residual squares covered a circular path (Figure 15A-B) that was centered inside of the EBL exposure field (Figure 15C). This implicated a known problem in which the Crestec EBL loses feedback control of its stage vertical position, resulting in loss of focus. This can be prevented by verifying that the Crestec’s focus laser properly identifies the patterned surface for each exposure field. Additional occurrences of this effect may be attributable to excessive electron beam current and hence overheating of chromium during evaporation. Proper centering and rastering of the beam on the source crucible will likely prevent this problem.

**Leaking grids**

A second class of quality problems was the occasional failure of grids to partition bilayers, which manifested itself as “leaks” during characterization with fluorescence recovery after photobleaching (FRAP). This often coincided with visible discontinuities in the grid lines themselves. Chromium is known to adhere very strongly to glass, and chromium films maintain their integrity despite aggressive sonication treatments. This suggested that surface contamination or incomplete lithography development prevented the adhesion of continuous chromium films on substrates.

SEM images of the resist film, sputtered with a thin gold-platinum conduction layer, showed many instances of contaminants embedded beneath the resist itself (Figure 16A-B). Glass substrates were typically cleaned with aggressive piranha treatment, expected to remove most organic contaminants. Furthermore, source glass coverslips were commonly observed to exhibit high dust content in their packaging. For this reason, the contaminants observed in SEM were suspected to be glass dust. By pre-treating substrates with Hellmanex II solvent (Hellma Analytics, Müllheim, Germany), a dedicated alkaline solution for glass cell cleaning, instances of this problem were greatly reduced.

Nonetheless, isolated discontinuities in chromium grids were still occasionally observed. Because resist development was performed outside of the clean room, the substrates were expected to be particularly vulnerable to contamination between the development and PVD operations. By carefully covering all work surfaces with clean Technicloth and minimizing exposure to air, this problem was almost completely eliminated.
Figure 16 – (A-B) Substrate contamination beneath ZEP-520A resist, coated with thin sputtered conduction film for imaging.

Figure 17 – Screen captures of Crestec electron beam spot patterned in ZEP-520A resist. Adjust stigmation to minimize beam size and maximize roundness (A). Gradual sample drift can be observed by tracking spot, and also causes a tail in the spot (B).
Exposure failure

Both hardware and software issues with the Crestec machine were observed to cause a number of problems, including partially exposed fields and excessive write times. Most importantly, lines must be exposed with a concentrated beam point spread function. Beam focus and stigmation were optimized by scanning test spots near the exposure area, confirming that the spot is concentrated and circular (Figure 17A). Weak adhesion of the substrate to the stage using aluminum tape also caused substantial drift, manifesting itself as a tail in the test spot (Figure 17B). These faults are readily diagnosable by following the Crestec manual procedures. To further mitigate the impact of these issues on pattern quality, exposure shot time was increased from 1.00 to 1.25 µs, providing a higher probability that a poorly focused exposure still results in a continuous resist pattern.

Some pattern files took an excessive time to write. If a pattern was predictably slow, exposure fields were often redundantly defined in the exposure file. This can be double-checked in the pattern writing software. Furthermore, exposure time appears to be limited by the cycle frequency of the beam blank, so grid lines were exposed much more quickly than an equivalent surface area of discontinuous features such as crosses. Finally, some pattern exposures sometimes unpredictably hung due to unknown software problems.

Preferred fabrication protocol

The currently preferred fabrication protocol for chromium grids is described in Table 1. The following comments on materials and procedures have been communicated to, and in some cases developed with, students Geoff O’Donoghue and Hiroyuki Kai to continue fabrication work.

Step 0: starting material

Grids were primarily fabricated on two types of circular glass substrates. Because the thickness range of #2 glass appeared to overlap the maximum substrate thickness for the Nikon 100x microscope objective, fabrication was conducted on #1.5 glass or thinner. Users of the Bioptechs flow cell system used 40 mm glass with approximate thickness rating #1.5 (0.16-0.19 mm) from the same supplier. Users of other fixtures predominately used 25 mm diameter glass from several suppliers. VWR and Fisherbrand both supply #1 glass in this dimension, although quality was frequently an issue. The edge of the glass was often jagged, and appeared to increase the substrate’s susceptibility to fracture during handling. Entire batches of glass often contained an unacceptable amount of dust as well, and this appeared to influence resist film uniformity at times. Warner #1.5 glass of 25 mm diameter was eventually purchased in large quantities from Harvard Apparatus (Hamden, CT), and this did not exhibit the same problems as the VWR and Fisher supplies. In all cases, these thin substrates are very fragile and require exceptional care during handling, especially during nanofabrication procedures that involve adhesion with vacuum-grade Kapton tape on flat surfaces.

Step 1: Soak substrates minimum 30 minutes in 1.25% Hellmanex II

The substrate cleaning procedure was amended to include a soak in dilute Hellmanex II at 40 °C owing to suspected dust contamination prior to resist coating. This product is an
alkaline solvent that includes emulsifiers to specifically removed surface-adsorbed contaminants from glass flow cells. Although the inclusion of this step is based only on anecdotal observations of improved corral continuity, the step is quick and inexpensive. Substrates were assembled in a Teflon rack specifically designed to carry coverslips (product #C-14784, Invitrogen / Life Technologies, Carlsbad, CA) and handled with stainless steel tweezers into the solvent bath. Following this procedure, substrates were rinsed thoroughly by transferring at least three times to beakers full of deionized water.

At the time of writing, Hellmanex II had been recently discontinued and replaced with Hellmanex III. Although there appears to be no major difference between the two products, the continued efficacy of this procedure has not been rigorously established with the new version.

Steps 2-5: Piranha clean minimum 5 minutes, rinse in water, rinse in isopropanol

The substrates were kept in their Teflon rack and transferred to a fresh “piranha” bath (3:1 sulfuric acid:hydrogen peroxide) for five minutes, then rinsed five times in fresh beakers of deionized water. This chemical reaction is hazardous, and this step must be carried out in the approved hood and follow the safety guidelines described in the laboratory’s piranha procedures. In particular, contact with the hot beaker and accidental contact with organic solvents must be carefully prevented.

Residual water can spread contaminants and adhere the substrates to their transport dishes during transport. To mitigate this problem, individual substrates were sprayed with isopropanol and dried with compressed nitrogen, taking care to push all liquid toward the perimeter of the substrate. To prevent dust contamination during resist spin coating, substrates were placed flat on clean TechniCloth (Connecticut Clean Room Corp., Bristol, CT) and covered with a clean quartz dish before transferring to Petri dishes or wafer carriers for storage. The TechniCloth absorbed any residual isopropanol on the edge of the substrate.

Steps 6-8: Deposit dilute Zeon Chemicals ZEP-520A electron beam

Zeon Chemicals ZEP-520A is an electron beam resist that exhibits high resistance to etching. It is also several times more sensitive to electron beam exposure than commonly used PMMA resist. Resist was diluted 1:3 in anisole. This resulted in a substantially thinner film at 1000 rpm, estimated to be approximately 50 nm in another user’s process. The described spin coating and exposure parameters were interpreted from LBNL Molecular Foundary staff. The resist has a rated expiration date of 12 months after shipping, although resists that were several years past this date did not show any deterioration in performance.

Because this film is extraordinarily thin, small dust contaminants can catastrophically disrupt the continuity of the resist layer. Polymer aggregation and settling is a common shelf-life concern with resists prepared in volatile solvents. To prevent film disruption from aggregates, the resist was deposited on the substrate using a Corning 0.45 μm PTFE syringe filter. Undeposited resist could then be deposited from the syringe back into the vial after removing the filter. After spin coating 40 sec at 1000 rpm with 500 RPM/sec ramp-up and ramp-down times, the film was soft-baked for five minutes at 145 °C on a hot plate covered in fresh aluminum foil. As dust contamination was quite prevalent, visual confirmation of a uniform, transparent resist film is advised.
Steps 9-11: Spin coat aquaSAVE conductive polymer

A conductive polymer was used to discharge the resist surface during EBL exposure. In typical devices fabricated with EBL, this is typically achieved by coating a silicon or glass substrate in a thin conductive film such as indium tin oxide (ITO). Because the surface composition of the substrate is critical for SLB assembly, however, this was not possible. Mitsubishi-Rayon aquaSAVE (Tokyo, Japan) is a convenient spin-on conductive polymer that is readily removable in water. However, it had not been possible to purchase the small quantities required for this application, and the laboratory supply has been a free sample from the manufacturer.

The polymer does not wet well to the underlying resist, requiring excessive quantities of aquaSAVE to be expended during coating. To conserve as much as possible, two or three drops of aquaSAVE were carefully deposited using a glass dropper into the center of the substrate, and gently dragged in a line to the edge of the substrate without touching the glass dropper directly to the underlying resist. After spin coating 40 seconds at 1000 rpm, this resulted in a conductive region that covered about a 5 mm circle in the center of the substrate, and that also extended a conduction path to its edge. This was sufficient to cover the typical EBL writing area and complete the necessary discharge circuit during EBL exposure.

Step 12: Electron beam lithography

Substrates were attached to the machine’s stage, either directly or via a four-inch dummy silicon wafer, using conductive aluminum tape. To achieve electrical continuity between the aquaSAVE film and device ground, the aluminum tape must at least contact the line of aquaSAVE from the substrate’s center to its perimeter. A minimum of three tape contact points per substrate are recommended to mitigate the substrates’ mechanical drift during the exposure process. As many as 12 substrates can be loaded in a single step and located in SEM imaging. By saving coordinates, the entire sample set can then be exposed as a batch process.

Electron beam exposure on the Crestec CABL-9510CC was nominally performed with a 600 μm x 600 μm fields at 60,000 x 60,000 dot resolution. Shot exposure was increased from 1.00 to 1.25 μs to mitigate potential underexposure problems. As the transparency of glass substrates sometimes caused erroneous height position measurements, special care should be taken to stabilize the stage height feedback laser, which should zero when the “wafer z” command is executed. If this proves difficult, moving the stage lateral position slightly and manually zeroing the stage height will eventually allow this height to home. After following the machine’s standard procedures for focus and stigmation adjustment, test spots should be exposed in the vicinity of the intended pattern area. These parameters should be fine-tuned to achieve spots that are small, round, and feature somewhat dark centers (Figure 17A). After exposure, log files should be saved for future estimates of exposure times, and to troubleshoot any problems that may arise.
Steps 13-15: Development

Between resist development and metal deposition steps, the substrate was particularly sensitive to dust contamination that could disrupt the continuity of grids. The conductive polymer was readily removed in dionized water, which was immediately dried from the written area of the substrate to prevent degradation of the resists. The resist was then developed by swirling in isoamyl acetate for 60 seconds, then cleaning and drying with isopropanol as usual. Exposure to dust should be minimized, and all contact surfaces cleaned with fresh Technicloth.

Step 16: Electron beam evaporation

The standard Edwards EB3 evaporation procedure was used, but must be precisely timed due to the very small deposition thickness. Chromium was evaporated between 0.8-1.5 nm/sec at an electron beam current of 28-31 mA. The shutter should be closed after approximately 4.5 nm have been deposited. The final QCM reading should be 6-7 nm.

Step 17-18: Metal film liftoff

Two baths of dichloromethane, both in an activated sonicator, were used to remove the resist. The substrate was rapidly submerged in the first bath and transferred immediately to the second, taking care to minimize the amount of time that it was exposed to air. The first bath was then removed and the chromium residue filtered from organic waste collection using a paper filter and funnel. The second bath was briefly removed from the sonicator, and covered in aluminum foil and then Parafilm to avoid contamination before sonicating for 10 minutes.

Completed grids were routinely inspected in 10x brightfield illumination prior to delivery for biological experiments.
Protein patterning with sacrificial barium fluoride films

Nanofabricated films partition supported lipid bilayers to mimic organized regions of cell membranes. A desirable extension of this method is the deposition of functional proteins in spatially organized regions. This could be used to simulate several naturally occurring structural motifs in cell membrane organization, including focal adhesions that recognize the glycoprotein fibronectin in the extracellular matrix.

Nanofabrication offers many opportunities to organize biological molecules at extraordinarily small spatial scales. However, these methods can introduce a variety of harsh conditions such as strongly acidic or basic solution, harsh organic solvent, surfactants and detergents, high temperatures, ultraviolet and X-ray irradiance, and high vacuum environments. Although suitable for nonbiological devices, these conditions generally preclude biological applications or limit the applicability of a technique to a very narrow class of biological molecule.

Many strategies have been developed to combine immobilized islands of protein in a mobile surface of supported lipid bilayer. Microcontact printing has been used to pattern protein features near 1 µm in size either before or after assembly of supported lipid bilayers. Fibronectin masked with aluminum thin films, which were subsequently dissolved in sodium bicarbonate (pH = 11.5), has also been successfully followed by support lipid bilayer assembly. However, these basic conditions are incompatible with most other proteins. A photoresist that dissolves in less aggressive aqueous condition has been used to pattern two different proteins via biotinylation atop a thin resist residue, but this would not present the bare glass surface necessary for SLB assembly.

A unique property of barium fluoride (BaF$_2$) thin films may be exploited to achieve patterning of proteins in supported lipid bilayers without introducing biomolecules to
harmful solvents. When patterned in thin films, BaF$_2$ is known to dissolve in water at a rate of approximately 1 nm/sec. However, the same film resists many aggressive solvents, including isopropanol, acetone, and dichloromethane. By engineering film thickness and SLB formation conditions, it is hoped that this can serve as a biocompatible sacrificial film (Figure 18).

The formation of fluid supported lipid bilayers is highly sensitive to the composition and cleanliness of the underlying silica substrate. To confirm that aqueous etching of barium fluoride did not disrupt this condition, test BaF$_2$ films were deposited on piranha-cleaned glass substrates and scratched with tweezers to establish a pattern boundary. Films 5-30 nm thick were deposited by electron-beam evaporation on the same Edwards EB3 as described in the chromium grids protocol. Deposition current was maintained at 4-7 mA to maintain a very slow rate of 0.1 nm/sec or lower. The film was then removed in filtered water and SUVs deposited on a plastic dish. SLBs were then formed by dropping the glass substrate onto the SUV drop, a common method for SLB assembly.

Fluorescent fibronectin labeled with AlexaFluor 647 was then added, and adsorbed preferentially to the scratched region (Figure 19). As expected from early studies of SLB patterning$^{56-58}$, the scratched glass was not fluid and did not recover when imaged with fluorescence recovery after photobleaching (FRAP) (Figure 20).

This proved that the deposition and subsequent removal of BaF$_2$ did not disrupt the surface cleanliness required to form stable supported lipid bilayers, and represents significant progress toward the patterning goal of this project. In order to control SLB and protein patterning, the etching kinetics of BaF$_2$ must be controlled while depositing SLB solution, which is expected to rapidly degrade the film. In addition to limiting the volume of SUV solution to less than 10 μL during SLB assembly, species transport in the etching reaction can be slowed by substituting a plastic dish for an atomically flat polished silicon wafer. Tuning the dilution ratio of SUVs in solution and the temperature of the SLB assembly process can also be used to optimize adsorption kinetics such that SLBs are only adsorbed to surfaces that were not covered in BaF$_2$. 

Figure 20 – Time sequence FRAP of SLB-NBD adsorbed during etching of sacrificial BaF$_2$ thin film. Fluorescence recovers on the main glass substrate but not in the horizontal scratch that is preferentially covered in fibronectin.
Single-molecule Ras assays with zero-mode waveguides

Background and motivation

Surface immobilization has driven many single-molecule fluorescence experiments that have contributed a tremendous amount of understanding to enzyme function, including highly publicized studies of F1-ATPase, myosin motor proteins, and dynein. Single-molecule methods are most commonly exploited to overcome limits in ensemble averaging of heterogeneity in the reaction rates of complicated enzymes.

Nanofabricated surfaces offer further control of surface chemistry, topology, and optical effects. The most striking example of this is the single-molecule real-time (SMRT) platform for DNA sequencing that is now a core technology of Pacific Biosciences (Menlo Park, CA). In SMRT, single polymerase molecules are tethered within zero-mode waveguides (ZMWs), cylindrical wells that confine optical excitation to a volume much smaller than that attainable with other common methods. In this case, single-molecule sensitivity enables real-time readout of the highly dynamic attachment of single nucleotides to a DNA template by DNA polymerases and changes in reaction kinetics associated with methylation.

ZMW have also been specifically applied to lipid membrane experiments. Lipid bilayer coating of ZMWs was used to investigate the mechanobiology of filapodia-like protrusions into small spaces. A fabrication protocol for planarized ZMW arrays has also been used to quantify membrane diffusion using fluorescence correlation spectroscopy.

Beyond the ability to differentiate single-molecule from ensemble-averaged kinetic, a similar strategy may address the challenge of characterizing single-molecule reaction rates in settings that are not simply limited by transport of substrates species. Many, if not most, enzymatic reactions of interest in signaling are only reaction-limited at substrate concentrations of micro- to millimolar. Of particular interest are putative variations in the nucleotide exchange of Ras by SOS, which may be linked to SOS structural fluctuations. In order to achieve single-molecule optical measurements at reaction-limited concentrations of Ras, optical observation volumes must be engineered far smaller than the limit posed by optical diffraction.

Figure 21 – Proposed experiment to characterize single Ras-SOS interaction at high nucleotide concentration. Image courtesy of Dr. Sune Christensen.
Table 2: Zero-mode waveguide fabrication protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Material</th>
<th>Qty</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Starting material</td>
<td>Circular coverglass, Warner #1.5 25mm</td>
<td>1</td>
<td>ea</td>
</tr>
<tr>
<td>1</td>
<td>Soak substrates minimum 30 minutes in 1.25% Hellmanex II (Helma GmbH, Muellheim, Germany)</td>
<td>Hellmanex II</td>
<td>1</td>
<td>mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Deionized water</td>
<td>80</td>
<td>mL</td>
</tr>
<tr>
<td>2</td>
<td>Piranha clean minimum 5 minutes</td>
<td>Deionized water</td>
<td>100</td>
<td>mL</td>
</tr>
</tbody>
</table>

*Warning: work with piranha only in dedicated hood and in accordance with SOP*

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Material</th>
<th>Qty</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Dip coverglass five times in deionized water, refilling water beaker each time</td>
<td>Deionized water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Rinse both sides with isopropanol squirt bottle</td>
<td>Isopropanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Dry with compressed nitrogen then keep substrate covered</td>
<td>Compressed nitrogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Tape substrates to 4” silicon carrier wafer in clean environment</td>
<td>Test-grade 4” Si wafer</td>
<td>1</td>
<td>ea</td>
</tr>
<tr>
<td>7</td>
<td>Load aluminum in nrc thermal evaporator</td>
<td>R.D. Mathis tungsten basket</td>
<td>1</td>
<td>ea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kurt Lesker 99.99% aluminum pellets</td>
<td>7</td>
<td>ea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quartz crystal</td>
<td>1</td>
<td>ea</td>
</tr>
<tr>
<td>8</td>
<td>Pump down to 5.0 - 10-6 Torr or lower</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Ramp power to 35 A current over the course of 1 minute</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Continue to ramp power up until spike occurs, usually in the 50-70 A range, and immediately decrease back to 35 A current</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Slowly increase power again until rate stabilizes at 1-2 Å/sec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Open shutter and deposit 100 nm aluminum, end process as normal, discard used basket</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Transfer substrates to SEM stub, affix using conductive tape</td>
<td>R.D. Mathis 16071 Al tape</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>FIB etch triangular alignment marks at 10 nA current</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>FIB etch ZMW arrays at 10 pA current</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Experimental plan**

ZMWs will be used to measure SOS exchanging single nucleotides on Ras-coated SUVs (Figure 21). By limiting the optical excitation volume, unit fluctuations in Ras-bound nucleotide fluorescence intensity will be observable despite high solution concentrations of labeled nucleotide. Entire ZMW arrays will be imaged in total internal reflection (TIRF) illumination for multiplex observation of many such SOS interactions.

**Fabrication**

ZMW arrays have been fabricated using focused ion beam (FIB) etching of aluminum films deposited with thermal evaporation. The fabrication protocol is summarized in Table 2 and details are described in this section.
Steps 0-5: glass substrate cleaning

Glass substrate selection and cleaning follow the same procedures as described for the fabrication of chromium grids. Warner 25 mm diameter #1.5 glass coverslips were favored, although Fisherbrand 18 x 18 mm #1 glass squares have also been used.

Step 6: tape substrates to carrier wafer

PVD equipment in the Marvell Nanolab requires nonstandard samples to be attached to 4” or 6” carrier wafers with Kapton vacuum tape. The larger wafer blocked the line of sight between metal source and quartz crystal monitor in the specific thermal evaporation equipment used in this protocol. For this reason, 4” wafers were used. Similar to EBL contamination concerns described in the previous section, dust contamination disrupted substrates at this stage, so samples were taped to their carrier wafers inside of the cleanroom.

Step 7-12: thermal evaporatoration of 100 nm aluminum

DC sputtering and electron-beam evaporation were both explored for aluminum evaporation, but thermal evaporation reliably produced high-quality films for this project. Aluminum films were deposited using the NRC thermal evaporator in the Marvell Nanolab. Aluminum source material is 0.125” diameter x 0.125” length, 99.99% purity aluminum pellets from Kurt Lesker Co. (#EVMAL40EXED, Clairton, PA). Tungsten wire baskets from R.D. Mathis (#B12B-3X.025W., Long Beach, CA) are 0.5” in diameter, 0.375” deep, and rated for 41 A current. A standard quartz crystal from the Nanolab supply room was installed. The quartz crystal monitor was set to density of 2.70 g/cm³, acoustic impedance (“z-value”) of 1.080 g/cm²s, and tooling factor of 141.9% determined by line-scan profilometry calibration.

Aluminum deposition is often difficult because it can quickly wet, alloy into, and fracture the tungsten basket. This was, however, mitigated by carefully controlling the evaporator power, rapidly responding to the melting of the aluminum, and discarding the basket after the process. After reaching the appropriate process vacuum pressure, the power control was ramped up until about 35 A current over the course of one minute. After this step, the power was continually increased until a rapid spike in current was observed, typically starting in the 40-50 A range and frequently exceeding 70-80 A. This rapid increase in conductivity between the process electrodes appears to be the melting of the aluminum pellets. As quickly as possible, reducing the power control and hence current back to about 35 A will stabilized the evaporation and minimized the risk of a basket failure. The power could then be gradually increased while observing the QCM reading and vacuum pressure until aluminum evaporation starts at about 1.5 Å/sec, finishing with an end film thickness of 100 nm.

Alumina-coated baskets (R.D. Mathis #RDM-WBAO-5) were also available and mitigated the failure modes of tungsten baskets, although the higher required current introduced substantial rate variability during evaporation. Stable deposition rates of 1.7-1.9 Å/sec were achieved at about 34 A with the uncoated baskets. Films were smooth and moderately reflective but had a distinct brown appearance. Further optimization of this process, or migration to electron-beam evaporation, is advised for aluminum deposition.
Step 13-15: focused ion beam etching

A variety of fabrication protocols have been developed for holes in metal films, and specifically for zero-mode waveguides. UV and electron beam lithography with negative-tone resists (e.g. Sumitomo NEB-31A, Tokyo, Japan), followed by anisotropic aluminum etching, appear to be the dominant fabrication approach for large-area patterns. Positive-tone electron-beam lithography was briefly explored during the early stages of this project. PMMA electron-beam resist displayed much improved adhesion to aluminum thin films (A2 PMMA, MicroChem, Newton, MA) compared to the ZEP-520A used for chromium grid fabrication. Wet aluminum etching in sodium hydroxide confirmed that positive-tone lithography sufficiently defined the ZMW profile through to the underlying aluminum, as evidenced by the definition of test holes and lines and isotropic profile of etching that extended far beyond these features (Figure 22A). Because of the inherent etch anisotropy required in this process, as well as poor PMMA resistance to chemical etching, wet etching was not chosen for further development. Due to equipment availability problems for anisotropic aluminum plasma etching, this EBL protocol was not pursued further.

Although capital-intensive and not sufficiently rapid for large-area fabrication, direct-write focused ion beam (FIB) etching offered rapid prototyping and simple dimensional control for ZMW arrays. For this reason, it has been adopted for diverse applications in laboratory-scale nano-optics and was chosen for further ZMW fabrication for this project. Etching was performed on a FEI Quanta dual electron and focused ion beam microscope installed in the Berkeley Biomolecular Nanotechnology Center.

Figure 22 – (A) Lithography test for ZMWs, lines and holes in PMMA-coated aluminum written with electron-beam lithography, then etched in sodium hydroxide (NaOH). Isotropic underetch profiles were expected and indicated completeness of resist development. (B) Alignment mark and ZMWs fabricated using direct-write focused ion beam (FIB) lithography.
Aluminum-coated samples were adhered to steel SEM stubs using conductive aluminum tape (R.D. Mathis #16071). Because ZMW arrays alone were difficult to identify in an optical microscope, triangular alignment marks were first etched at high FIB current (Figure 22B), nominally 10 nA. ZMW patterns were defined as 100 nm diameter circles in arrays with 5 µm spacing, using the default FIB spot pitch of 6.5 nm. The Quanta FIB system’s two lowest FIB currents are 1.6 and 10 pA, typically used for FIB imaging although sufficiently strong to etch aluminum at the dimensions of ZMWs. Pattern definition and etch time were substantially superior with the 10 pA etch time. Etch time per ZMW was optimized with optical measurements described later.

Sample mounting is still somewhat problematic in the FIB segment of this protocol. The aluminum tape fails at times to rigidly adhere substrates to stubs, and drifting on the order of 1 µm/sec. can occasionally be observed in both SEM and FIB images. While other users can use two-sided carbon tape for adhesion and conductive contact, the fragile nature of these #1.5 glass substrates presents a high risk of sample fracture during handling.

Furthermore, charging has recently become a problem in aluminum films that were-shadow masked to 3 x 3 mm patches during deposition, despite multiple points of electrical contact between the tape and the aluminum. This manifested itself as a rapid depletion of signal in both SEM and FIB detectors after prolonged FIB exposure, and was vastly worse at higher FIB currents. Although the detectors’ brightness was restored within a minute after interrupting the FIB beam, this effect made fabrication time-consuming and may have imposed other adverse conditions on the sample. For this reason, aluminum should be deposited to cover the majority of each substrate, or otherwise a conductive polymer or similar strategy discharging strategy should be employed. Dedicated SEM stubs with integrated copper clips may also be an attractive alternative for both mechanical and electrical contact in FIB.

**Optimization of ZMW etch dose**

Because of the very small dimensions of ZMW arrays, precise characterization of hole depth was difficult with direct SEM observation. Instead, FIB doses for ZMW patterning were optimized using brightfield imaging.

First, to gain a coarse estimate of aluminum etching rates, a series of 5 µm diameter calibration circles were etched at 10 pA into a 100 nm aluminum film and imaged in brightfield with a Nikon 10x air objective (Figure 23A). Because these holes were much larger than ZMWs, attenuation of transmitted optical modes was not a significant effect, and transmission was a good proxy for completeness of the aluminum etching process. As etch time and hence dose were increased, hole transmission abruptly changed from 0 to nearly 100%. Remaining film thickness \( k \) was then estimated from the brightfield transmission \( T \) using an effective attenuation coefficient:

\[
\frac{T}{T_{\text{max}}} = e^{-\frac{k}{\alpha}} 
\]  

Eq. 4
where aluminum skin thickness $\alpha$ was estimated from published complex refractive index data$^{91}$ to be 3.5 nm. Because of this high attenuation, any gray transmission must correspond to a relatively narrow range of film thicknesses. Hence the single partially transmissive hole corresponding to 15 sec etch time was still nearly etched to completion at 10 pC/µm$^2$ FIB dose, corresponding to 8 ms etch time per 100 nm diameter ZMW at 10 pA. Complete etching should therefore occur at 10-15 pC/µm$^2$ dose.

Brightfield imaging of test ZMW arrays, however, showed partial (gray) transmission at a significantly higher dose (Figure 23C) around 64 pC/µm$^2$ in brightfield imaging with a 100x oil immersion objective. This could be due to two effects. First, a broader area than 100 nm was surely etched for a nominal 100 nm FIB scan diameter, which likely exceeds this scan boundary due to both well-known draft angle effects and the finite size of the FIB point spread function. Second, small holes such as ZMWs truncate guided modes gradually, so the cumulative white-light intensity transmission through ZMWs may be subject to the same reduced density of propagating modes that ultimately lead to near-field optical confinement. Although images are grayscale only, this hypothesis would be supported by an observable blue shift in the white-light transmission.

Indeed, because ZMWs are observable at all in these brightfield images implies that their diameters are not sufficiently small to achieve exclusively evanescent excitation and hence maximize rejection of background. Although not immediately apparent from brightfield transmission, these arrays may be somewhat overetched into the glass substrate, a condition that does not compromise optical performance and indeed has been demonstrated to improve SNR when properly engineered$^{92}$. Further optimization of this etch dose in a narrower range would be especially aided by a convenient means of characterizing the depth of ZMWs, for example using atomic force microscopy (AFM).
Substrate fixture and passivation chemistry

Initial experiments in which 25mm ZMW substrates were mounted in stainless steel “donut” fixtures often ended with abrupt dissolution of the aluminum film, a failure that was attributed to galvanic corrosion between these two metals. This was mitigated by encapsulating the ZMW active surfaces in poly(dimethyl siloxane) (PDMS) flow cells or by substituting the steel fixture with an equivalent version machined from Teflon, increasing the thickness of the bottom shelf and O-ring groove to accommodate the softer material (Figure 24). Because of the low friction of Teflon, the fixtures need to be very carefully assembled so as not to damage the threads.

In order to prevent nonspecific adsorption of SUVs and nucleotide to aluminum, and the resulting depletion of reactants in the ZMW cavity, a surface passivation strategy needs to be optimized alongside SUV immobilization chemistry. Studies using poly(vinylphosphonic acid) (PVPA) passivation and poly(L)lysine-polyethylene glycol SUV attachment on test aluminum films are underway.

Figure 23 - (A) Brightfield transmission through FIB test patterns, (B) Peak transmission v. dose calibration used to calculate ZMW etch time, (C) Dose test through ZMW arrays, brightfield in 100x oil immersion microscope.
Figure 24 – Drawing of Teflon “donut” fixture for 25 mm diameter coverglass.
**ZMW preliminary results**

ZMW arrays treated with BODIPY-labeled SUVs showed individual points of fluorescent emission that corresponded to peaks in brightfield transmission (Figure 25A). Atto647N-labeled SOS emitted in a higher number of locations in its corresponding emission channel, suggesting that SOS adsorbs nonspecifically inside ZMWs that are not occupied by SUVs (Figure 25B). This necessitates further optimization of substrate passivation and SUV attachment chemistries. Closer observations of adsorbing SUVs show many observations of step intensity fluctuations, demonstrating that single SUVs adsorb and detach into the ZMW cavity with lifetimes on the order of 10 sec (Figure 25C-D).

**Conclusions**

These projects have advanced nanofabrication strategies that engineer the diffusive landscapes of reconstituted lipid systems. Chromium grids continue to be a valuable tool with which to partition fluid SLBs. Improvements in fabrication yield and repeatability have allowed this approach to be integrated into more sophisticated experiments, including cell experiments that require large surface areas of homogenous grid quality. A related protocol, in which water-soluble BaF$_2$ was used to assemble defined fields of fluid SLB, will be highly useful to integrate well-defined clusters of immobile proteins when simulating intercellular interactions. The utility of zero-mode waveguides in single-molecule enzyme measurements has already been demonstrated in the context of DNA sequencing, and promises to further advance kinetic characterization of membrane protein reactions at the single-molecule level. In all cases, precise control and optimization of nanolithography and film deposition are instrumental in achieving this unsurpassed manipulation of biomolecules’ environments.
4. TIME-RESOLVED FLUORESCENCE AT SUBMICRON RESOLUTION

Introduction

Molecules diffuse randomly in both translational and rotational coordinates. Translational diffusion, as measured by fluorescence correlation spectroscopy (FCS) and fluorescence recovery after photobleaching (FRAP), is a ubiquitous parameter of biological membranes, and frequently used to infer information about membrane proteins’ viscous microenvironments and interactions. However, inferences based on translational diffusion must consider the complicated and often unquantifiable scaling of translation drag in membranes. Although rotational diffusion offers a far simpler relation between a protein’s diffusion and interactions, the limited lifetimes of most common fluorescent molecules make precise quantification of rotation in membranes with time-resolved fluorescence anisotropy (TRFA) a difficult goal.

The sources of noise and fitting uncertainty are explored here, and the advantages of TRFA on fluid supported lipid bilayer (SLB) systems discussed. A photon-counting hardware system capable of measuring TRFA and FCS is then described, as well as the supporting software needed to process multiple spectroscopy analyses from photon-counting hardware. Operating protocols for both processes are included for future users of this system. Finally, system performance is established on a number of control samples prior to elaborating on its implementation in several major membrane projects in following chapters.

Fluorescence anisotropy and rotational correlation

Fluorescent molecules are electrical dipoles, and absorb and emit light along distinct vectors. These two dipole orientations are determined by the polarity of the molecular excited state, and are close to coincident in most common fluorophores.

Emission can therefore be biased toward a specific polarization direction by aligning the transmission vector of polarizers in the illumination and emission paths. This condition, called photoselection, results in the maximum modulation of fluorescence polarization immediately following the illumination pulse. In fluid specimens, rotational diffusion then causes fluorescence to depolarize toward a fully mixed state with no polarization preference.

This is classically expressed using fluorescence anisotropy $r$ in terms of emission intensity components parallel and perpendicular to the polarization direction of illumination:

$$r_{ideal} = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

Eq. 5

which represents the ideal physical behavior without experimental differences in the measurements of the two intensity components. This describes the difference in the intensities between the polarized direction and its perpendicular component, normalized by the total intensity of the sample:
Total intensity counts the perpendicular component twice because the electric field component parallel to the propagation vector of light decays from the photoselected orientation, but is not collected in a collimated optical system.

In general, separate measurements of these two components are performed either in series, by rotating the orientation of the illumination polarizer, or in parallel by splitting the emitted beam by polarization to two separate photodetectors. Both of these conditions introduce an additional calibration parameter $G$ such that

$$r = \frac{I_\parallel - GI_\perp}{I_\parallel + 2GI_\perp} \quad \text{Eq. 7}$$

and the total intensity is similarly adjusted:

$$T = I_\parallel + 2GI_\perp \quad \text{Eq. 8}$$

Conversely, the intensity components can be described by:

$$I_\parallel = \frac{T}{3}(1 + 2r) \quad \text{Eq. 9}$$

$$I_\perp = \frac{T}{3G}(1 - r) \quad \text{Eq. 10}$$

In most common biological experiments, anisotropy decays at a similar timescale as the photophysical interactions of the fluorophore itself. Hence the time dependence of the total intensity frequently figures into the physical interpretation of anisotropy in both steady-state and time-resolved experiments.

**Viscous flow relations**

Fluctuation-dissipation theory predicts that the same viscous forces resist displacement in forced and freely diffusing systems. Analogous versions of the Einstein-Smoluchowski theorem relate kinematic descriptions of random diffusion to a viscous drag term $f$ and Boltzmann factor $k_BT$. For translational diffusion:

$$\frac{\langle x^2 \rangle}{t} = \frac{k_BT}{f_T} \quad \text{Eq. 11}$$

for time-averaged mean square displacement $x$. Similarly, rotational diffusion is related to an analogous drag factor:
\[ \frac{\langle \theta^2 \rangle}{t} = \frac{k_B T}{f_R} \]  
Eq. 12

where \( \theta \) describes angular displacement. Crucially, viscous drag in random diffusion is equivalent to that of a similarly moving particle with the same relative velocity field.

In general, viscous interactions in a velocity flow field \( \mathbf{v} \) are governed by mass conservation and the Navier-Stokes equations for momentum:

\[ -\nabla p + \eta \nabla^2 \mathbf{\dot{v}} = \rho \left( \frac{\partial \mathbf{\dot{v}}}{\partial t} + \mathbf{\dot{v}} \cdot \nabla \mathbf{\dot{v}} \right) \]  
Eq. 13

for scalar fluid density \( \rho \) and viscosity \( \eta \), and pressure \( p \) that generally varies in space. In the case of highly viscous flow, such as conditions encountered in molecular and cell biology, inertial effects are negligible and the right-hand side of Eq. 15 becomes zero. Viscous interactions are then subject only to Stokes flow:

\[ \eta \nabla^2 \mathbf{\dot{v}} = \nabla p \]  
Eq. 14

Mobile objects then experience drag that can be computed from surface-integrated viscous shear force after solving for the flow field.

**Stokes spherical rotational correlation**

The most basic model in time-resolved anisotropy is that of an effectively spherical molecule of radius \( R \) that decays in a solution of viscosity \( \eta_{\text{soln}} \) with a single-exponential component \( \tau_{r_1} \):

\[ r(t) = r_0 e^{-t/\tau_{r_1}} \]  
Eq. 15

where the time constant is defined as the rotational correlation time (RCT) and follows directly from Stokes flow rotational drag applied to the rotational Einstein-Smoluchowski relation:

\[ \tau_{r_1} = \frac{4}{3} \pi R^3 \eta_{\text{soln}} \]  
Eq. 16

The initial anisotropy \( r_0 = 0.4 \) in the case of perfectly unhindered three-dimensional diffusion with coincident fluorophore absorption and emission dipoles. Provided \( r_0 \) is known in advance and total lifetime decays with a single exponential component \( LT \), rotational correlation can be calculated from steady-state anisotropy \( r \). Time integration of Eq. 15, weighted for decaying total intensity, yields the well-known Perrin equation:

\[ \frac{r_0}{r} = 1 + \frac{LT}{\tau_{r_1}} \]  
Eq. 17
where \( r \) is a time-independent measurement. However, time-resolved measurements are still necessary to prove that anisotropy decays with a single exponential component, a condition that often does not hold in labeled biological molecules.

**Diffusion in membranes**

The translational diffusion of lipid molecules in fluid bilayers has been addressed by a variety of scale-dependent fluid models\(^9^5-^9^8\) and follows no single simple solution to Eq. 14. Saffman and Delbrück attempted to reconcile this paradox by considering the effects of finite membrane thickness and interactions with the viscosity of the surrounding medium, assuming that membrane viscosity \( \eta_{\text{lipid}} \gg \eta_{\text{water}} \). This relation shows only a weak relation between translational diffusion coefficient and particle radius:

\[
  f_{T,2d} = \frac{4\pi \eta_{\text{lipid}} h}{\log \left( \frac{\eta_{\text{lipid}} h}{\eta_{\text{medium}} a} \right) - 0.5772}
\]

Eq. 18

Gambin and colleagues\(^9^8\) compared their own FRAP data as well as previously published scaling of translational mobility prediction from the Saffman-Delbrück equation. Bacteriorhodopsin was compared to a control polypeptide standard, and the slow mobility observed would have only been attainable with a Saffman-Delbrück radius that would have been easily observable in microscope. They concluded that proteins and other molecules that are on the same size scale as the lipid cross-sectional dimensions show a stronger empirical relation between diffusion coefficient and size:

\[
  f_{T,2d} \propto 4\pi \eta_{\text{lipid}} ha
\]

Eq. 19

Although the constant of proportionality in this relation varied between reported experiments.

Numerous investigations of translational diffusion in membranes, using techniques such as FCS and FRAP, reinforce the principle that this variable cannot be easily computed from basic protein parameters, and instead is affected by complex chemical effects\(^9^9,^1^0^0\). However, as reported by Saffman and Delbrück, viscous resistance to rotational motion on a cylindrical body of radius \( r \) and height \( h \) is readily calculated in a two-dimensional membrane of viscosity \( \eta_{\text{lipid}} \):

\[
  f_{\text{lipid}} = \frac{2}{3} \pi hr^2 \eta
\]

Eq. 20

This scaling is the basis of the following investigations into protein rotational diffusion in cell membranes.
Review of fluorescence anisotropy methods

By titrating temperature and solvent viscosity, steady-state anisotropy analysis using the Perrin equation (Eq. 27) has long been used to characterize gyration radii and conformation changes in a diversity of molecules\textsuperscript{11}. More recent applications of time-independent fluorescence anisotropy include fluorescent aptamer-based biosensors\textsuperscript{101}, in which a short nucleic acid sequence is evolved against protein or small molecule targets\textsuperscript{102,103}. Anisotropy of surface-tethered aptamer arrays has been measured using total-internal reflection microscopy, for example to array for growth factors and other common cancer biomarkers\textsuperscript{104,105}. Because the anisotropy signal measures slowed rotational diffusion of the aptamer-analyte complex, secondary binding strategies have been developed to amplify the mass signal of small molecule analytes such as ATP and cocaine\textsuperscript{106}. Because aptamers adapt highly specific shapes to their target molecules, the anisotropy signal can be localized to individual aptamer bases, and total static anisotropy can be maximized by judicious selection of fluorophore binding site. This effect can even be used to effect a decreases in aptamer-target anisotropy in specific cases\textsuperscript{107}.

Anisotropy can exhibit two-exponential decay due to a mixture of two species of single-exponential decays $\tau_{RA}$ and $\tau_{RB}$, in which case the ensemble decay behaves as a linear combination. Alternatively, a single species can result multi-exponential anisotropy as a result of multiple simultaneous modes of rotational diffusion. A simple case is the three-dimensional tumbling of an elongated molecule, whereby rotation about its longitudinal axis occurs much more quickly than about an orthogonal axis. This single-species, bimodal model is also observable in cases where the fluorophore diffuses relative to the molecule center of mass via a flexible subunit\textsuperscript{11}.

TRFA has been widely applied to proteins in solution. For example, TRFA has been used to quantify changes in the RCT of GFP with the composition of its medium or upon antibody binding\textsuperscript{108,109}. Ligand binding of solubilized protein receptor affinity domains has been inferred from multicomponent depolarization of labeled neurotoxins\textsuperscript{110}. Indeed, multicomponent TRFA can be used to differentiate bound and unbound fractions of such small molecules, and as such can theoretically be used as a diffusivity filter in time-resolved measurements.

Early studies of rotational diffusion in membranes using steady-state fluorescence anisotropy suggested that lipid-specific molecules were essentially immobile on the timescales of fluorescence lifetime\textsuperscript{111,112}. Time-resolved measurements provided a more nuanced view of this environment, however, as anisotropy often decayed at a relatively rapid rate but only to a nonzero limit that was attributed to independent probe tumbling within a limited solid angle\textsuperscript{113,114}. This was confirmed by theoretical consideration that quantified the contribution of tumbling to these limiting anisotropies\textsuperscript{115}, as well as changes in rotational diffusion following detergent solubilization of the membrane\textsuperscript{116} and cholesterol treatment\textsuperscript{117}.

Multicomponent TRFA decays have been related to multiple sources of structural flexibility in proteins, and can be predicted from corresponding viscous models\textsuperscript{11,118,119}. Time-resolved anisotropy of ethidium bromide and other intercalating dyes can also show a “dip and rise” profile, i.e. an increase in anisotropy during the nanoscale decay timeframe because both the dye’s lifetime and its rotational correlation increase drastically upon
intercalation in to DNA\textsuperscript{120,121}. Consideration of this effect was necessary to monitor the reduced torsional flexibility of DNA in cells\textsuperscript{122}.

Homo-FRET is a specific case of Förster resonance energy transfer (FRET), in which donor emission and acceptor excitation bands overlap. By definition, homo-FRET between adjacent fluorophores results in no measureable difference in rate of radiative emission, but can be detected by its signature rapid dopolarization. TRFA has been used to identify strong homo-FRET in aggregated fluorescent protein-lipid anchor complexes, and time-independent anisotropy imaging later used to localization naturally occurring protein clusters in live cells\textsuperscript{123,124}. Homo-FRET has also been used in conjunction with fluorescence correlation to quantify the number of CamK2 subunits that incorporate into cytosolic oligomers\textsuperscript{125}.

Efficiency of homo-FRET is highly sensitive to the relative orientation of fluorophore dipoles, and perfect alignment is rare in biological systems. For this reason, homo-FRET must often be compared to models that address the stochastic orientation distribution in proteins or protein aggregates\textsuperscript{126}. Home-FRET between large ensembles of neighboring fluorescence molecules was compared to complicated models of the orientation distribution of monomeric actin in fiber, and used to infer structural properties of F-actin labeled site-specifically with BODIPY\textsuperscript{127}. Multiparameter fluorescence detection (MFD) schemes have been developed in which pairs of variables, such as lifetime and anisotropy, are simultaneously correlated during single-molecule fluorescence bursts\textsuperscript{128–131}. Anisotropy has also been used to identify homo-FRET orientations in single molecules, for example the identification of three distinct DNA binding sites in reverse transcriptase\textsuperscript{132}.

Rotational diffusion and dipole anisotropy are also heavily researched effects in magnetic resonance spectroscopy and imaging. Rotational correlations contribute to resonance in protein NMR, and in fact configuration of measurements to remove polarization artifacts are a routine part of many protocols. This analogy has been explored in the context of weakly fluorescent tyrosine and tryptophan residues, the rotational correlations of which were proposed to be useful for deciphering protein structures\textsuperscript{133}. Although time-resolve depolarization measurements such as those performed with fluorescence are not feasible, sophisticated theoretical models have been developed to control for their effects, and may prove useful for more complicated applications of fluorescence anisotropy in biological systems.

**Recovery of rotational correlation models from TRFA data**

**Uncertainty in empirical anisotropy traces**

Time-resolved fluorescence anisotropy is measured using photon-counting techniques, predominately time-correlated single-photon counting (TCSPC), that assign photon arrival counts $I(t)$ to $m$ synchronous bins of width $\Delta t = t_{i+1} - t_i$. Repetition of this cycle many times assembles effective time-resolved intensity responses to the excitation results, in which fluorescence typically matches single- or multi-exponential time decays, each with a corresponding fluorescence lifetime $\tau_{LT}$ on the order of nanoseconds. Anisotropy is calculated from two such fluorescence emission traces that both obey Poisson statistics in each TCSPC bin, namely that the variance and average count numbers in a given bin are equal:
\[ \sigma_{\parallel,i}^2 = I_{\parallel,i}\Delta t \]  
Eq. 21

\[ \sigma_{\perp,i}^2 = I_{\perp,i}\Delta t \]  
Eq. 22

Because measurements are independent, random errors are independent, and the variance in anisotropy can then be calculated by propagating these errors in quadrature from Eq. 21-Eq. 22:

\[ \sigma_{r,i}^2 = \left( \frac{\partial r}{\partial I_{\parallel}} \right)^2 \sigma_{\parallel,i}^2 + \left( \frac{\partial r}{\partial I_{\perp}} \right)^2 \sigma_{\perp,i}^2 \]  
Eq. 23

\[ \frac{\partial r}{\partial I_{\parallel}} = \frac{1}{I_{\parallel} + 2GI_{\perp}} - \frac{I_{\parallel} - GI_{\perp}}{(I_{\parallel} + 2GI_{\perp})^2} \]  
Eq. 24

\[ \frac{\partial r}{\partial I_{\perp}} = \frac{-G}{I_{\parallel} + 2GI_{\perp}} - \frac{2G(I_{\parallel} - GI_{\perp})}{(I_{\parallel} + 2GI_{\perp})^2} \]  
Eq. 25

Which can be simplified in terms of the anisotropy function \( r(t) \) and total intensity \( T(t) \):

\[ \frac{\partial r}{\partial I_{\parallel}} = \frac{1}{T} (1 - r) \]  
Eq. 26

\[ \frac{\partial r}{\partial I_{\perp}} = \frac{-G}{T} (1 + 2r) \]  
Eq. 27

Leading to

\[ \sigma_{r,i}^2 = \frac{1}{T} \left( \frac{1 + G}{3} + Gr - r^2 + \frac{2 - 4Gr}{3} r^3 \right) \]  
Eq. 28

This expression for the variance in anisotropy is then applied to weighted-residuals nonlinear-least squares fitting that seeks to minimize

\[ \chi^2_R = \frac{1}{m - v} \sum_{i}^m \frac{(r_i - r_{mod,i})^2}{\sigma_{r,i}^2} \]  
Eq. 29

Anticipated anisotropy standard deviation \( \sigma_r(t) \) was simulated for a number of experimental parameters. Uncertainty is highly dependent on both the fluorescence lifetime and peak number of photons in the TCSPC total intensity (Figure 26A-B). However, calibration \( G \)-factor and the rotational correlation time itself do not drastically influence uncertainty in this parameter across a broad range (Figure 26C-D).
Figure 26 – Poisson noise contribution to standard deviation in anisotropy $\sigma_r$ due to (A) fluorescence lifetime, (B) peak TCSPC photon counts, (C) calibration $G$-factor, (D) rotational correlation time.

Figure 27 – Confirmation of anisotropy standard deviation $\sigma_r$ in (A) SLB-tethered YFP-ICAM-1. Nonlinear least-squares fit (solid black line) is superimposed on the same experimental anisotropy decay (blue dots), binned from native resolution of 4 ps (bin factor = 1) up to 128 ps (bin factor = 16). Standard deviation bounds (black dashed lines) correlate strongly with observed variance in data from least-squares fit, and bound 50-60% of all data points.
Furthermore, Eq. 28 accurately predicts the variation in real anisotropy decay data about its least-squares fit. Yellow-fluorescent protein (YFP) attached to ICAM-1 on supported lipid bilayers (Figure 27A) decays with a rotational correlation time of 28-30 ns. The predicted \( \sigma_r(t) \) matches the spread in data points from the least-squares fit, which increases with time due to the nanosecond decay of fluorescence. This applies across a range of time resolution from 4-128 ps obtained by binning TCSPC channels by factors of two (Figure 27B-F). The standard deviation bounds 50-60\% of data points in each trace, only slightly narrower than the 68\% predicted from Gaussian-distributed noise at each TCSPC channel.

**Predictions of parameter uncertainties**

Uncertainty of fitted parameters in anisotropy decay analysis can be predicted independent of any specific fit algorithm. This models the repeatability of specific anisotropy fitting routines in experiments with specified lifetime decays, and also helps evaluate the confidence in parameter values from specific fit models. For a general anisotropy decay model with \( \nu \) parameters:

\[
r_{\text{mod},i} = f(p_1, \ldots, p_\nu)
\]  
Eq. 30

The same error propagation technique from Eq. 23-Eq. 25 can be applied to calculate the uncertainty in a given fit parameter \( p_j \):

\[
\sigma_{p_j}^2 = \sum_i \left( \frac{\partial p_j}{\partial r_{\text{mod},i}} \sigma_{r_{\text{mod},i}}^2 \right)^2
\]  
Eq. 31

where the partial derivatives are calculated directly from the discretized definition of the fit model, and the anisotropy variance function is known from above. In multi-parameter models, explicit expressions for the parameter uncertainty are only attainable if parameters are independent variables, i.e.

\[
\frac{\partial p_j}{\partial p_k} = \begin{cases} 
1 & \text{if } j = k \\
0 & \text{if } j \neq k 
\end{cases}
\]  
Eq. 32

This expression for parameter uncertainty can also be simplified as the inner product of two vectors of length \( m \):

\[
\sigma_{p_j}^2 = \vec{M} \cdot \vec{E}
\]  
Eq. 33

where the first vector \( \vec{M} \) is calculated directly from the model definition and the second \( \vec{E} \) from a description of intensity traces. In the absence of actual experimental results, the second vector can incorporate estimates of fluorescence intensities and the calibration factor \( G \). The first vector can also be formulated in terms of the fit Jacobian.
considering that actual experimental observations $r_i$ are invariant and hence removed from the derivative. The Jacobian matrix is implicitly used to accelerate convergence of common nonlinear regression techniques, including the Levenberg-Marquardt algorithm, used in subsequent analysis.

**Parameter uncertainties in general one-exponential anisotropy decay**

Although a simple model, Eq. 15 represents a common time-resolved anisotropy measurement in which the rotational correlation time is desired within a given precision. Although $r_0$ is frequently a dependent variable of interest in experiments, and can be lower in ensemble experiments when dipole vectors and system illumination are not perfectly aligned, the uncertainty of the rotational correlation function in perfect solution measurements is of particular interest here.

This derivation will determine the uncertainty, and implicitly the slowest measureable rotational diffusion, for several key experimental variables: the rotational correlation time $\tau_{r1}$ itself, the time-dependent total fluorescence intensity $I_{tot}(t)$, the calibration factor $G$, and the number $m$ and spacing $\Delta t$ of TCSPC bins $t_1, ..., t_m$.

Expressing the model anisotropy decay function in the form of Eq. 30:

$$r_{mod,i} = p_1 e^{-t_i/p_2}$$  \hspace{1cm} \text{Eq. 35}

The model vector $M$ is computed from implicit differentiation for $p_2$:

$$\frac{\partial r_{mod,i}}{\partial p_2} = p_1 \left( \frac{t}{p_2^2} \right) e^{-t_i/p_2} + \frac{\partial p_1}{\partial p_2} e^{-t_i/p_2}$$  \hspace{1cm} \text{Eq. 36}

From Eq. 32, model parameters are assumed to be independent, hence eliminating the second term. Inverting the derivative leads to the partial derivatives in model-dependent vector $M$ (Eq. 33):

$$\frac{\partial p_2}{\partial r_{mod,i}} = \frac{p_2^2}{t_i p_1 e^{-t_i/p_2}}$$  \hspace{1cm} \text{Eq. 37}

which can also be formulated to include the complete model anisotropy decay function $r_{mod}(t)$:

$$\frac{\partial p_2}{\partial r_{mod,i}} = \frac{p_2^2}{t_i r_{mod,i}}$$  \hspace{1cm} \text{Eq. 38}

The experiment-dependent vector $E$ is then taken directly from Eq. 28.
Fluorescence lifetime limitations to rotational correlation

The decay of fluorescence excited state population through radiative and nonradiative pathways imparts a limited memory of orientation state at the instant of excitation. In the familiar scenario of photon emission histograms displaying single- or multi-exponential decay, emission intensity decays according to the fluorescence lifetime. Hence the ratio of rotational correlation time to fluorescence lifetime is the figure of merit in the sensitivity of fluorescence anisotropy to slow rotational diffusivity characteristic of proteins.

Numerous approaches have been adapted to overcome this limitation. Time-resolved anisotropy of phosphorescence emission, which exhibits lifetimes that are several orders of magnitude slower than typical fluorescence systems, has been used to study the rotational diffusivity of immunoglobulin binding to cell-surface receptors. Time-resolved monitoring of triplet state absorption can be used to monitor even slower rotational diffusion, on the order of millisecond, on transmembrane proteins that are fully surrounded by the viscous drag of the membrane itself. Polarization-resolved fluorescence correlation has been used to identify changes in nanorod hydrodynamics due to surface peptide binding, and a similar method can be applied directly to time-tagged photon streams.

Hardware design

Optical path

A system for time-resolved fluorescence spectroscopy, including anisotropy, has been developed and is described schematically in Figure 28. The optical path describe here was adapted from a confocal illumination and detection system used for fluorescence cross-
correlation spectroscopy (FCCS), and is capable of single-color TRFA as well as FCCS using three photodetector channels.

A tunable MaiTai Ti:sapph laser provides femtosecond pulsed illumination that pumps a tunable Inspire optoparametric oscillator (OPO) at 970 nm base wavelength (Spectra-Physics / Newport, Santa Clara, CA). Fluorophore excitation can be tuned from 490-640 nm using OPO cavity resonance, or alternatively set to approximately 485 nm by directing the direct single-harmonic generation (SHG) beam from the Inspire and changing the IR laser resonance. A Pockels cell and countdown electronics (ConOptics, Danbury, CT) are used to synchronize to the laser mode-lock signal and used to pick pulses to reduce illumination pulse frequency. Additional 488 nm picosecond pulsed illumination can be included with a pulsed diode laser (PicoQuant, Berlin, Germany) for two-color experiments.

Both laser lines are combined in a single-mode optical fiber that provides a visibly first-order Gaussian beam at the expense of substantial power loss. The excitation beam is then directed through a rotatable linear polarizer to a periscope mirror pair before entering a Nikon Eclipse inverted microscope (Nikon Instruments, Tokyo, Japan) with a perfect-focus system (PFS) to maintain high focus precision for long exposures. A 25x36 mm dichroic beamsplitter on a custom adjustable mechanical mount then reflects the lateral beam upward into the back aperture of a Nikon infinity-corrected objective lens, typically a 100x TIRF oil-immersion objective. The dichroic only reflects key laser wavelengths (405/488/561 nm or 405/640 nm) and transmits emitted fluorescence before entering the microscope’s filter wheel. An additional filter cube rejects the aforementioned laser wavelengths (488/641) to further reduce excitation beam contamination in the fluorescence emission signal.

Figure 29 – Detailed optical configuration of photodetector module.
Table 3 – Instrument connections for fluorescence spectroscopy

<table>
<thead>
<tr>
<th>Unit A</th>
<th>Label</th>
<th>Unit B</th>
<th>Label</th>
<th>Cable</th>
</tr>
</thead>
<tbody>
<tr>
<td>MaiTai</td>
<td>40 MHz output</td>
<td>ConOptics 305</td>
<td>External input</td>
<td>BNC</td>
</tr>
<tr>
<td>ConOptics305</td>
<td>Analog output</td>
<td>ConOptics 25D</td>
<td>Signal input (rear)</td>
<td>BNC</td>
</tr>
<tr>
<td>ConOptics305</td>
<td>Sync output</td>
<td>DB64 (rear unit)</td>
<td>Input</td>
<td>BNC</td>
</tr>
<tr>
<td>DB64 (rear unit)</td>
<td>Output</td>
<td>PDL 800-B</td>
<td>Trigger in</td>
<td>BNC</td>
</tr>
<tr>
<td>PDL 800-B</td>
<td>Sync out</td>
<td>DB64 (front unit)</td>
<td>Input</td>
<td>BNC</td>
</tr>
<tr>
<td>DB64 (front unit)</td>
<td>Output</td>
<td>PH300 CTRL</td>
<td>Proprietary</td>
<td>SMA</td>
</tr>
<tr>
<td>PHR 800</td>
<td>PH300 Channel 1</td>
<td>PH300</td>
<td>Channel 1</td>
<td>SMA</td>
</tr>
<tr>
<td>PHR 800</td>
<td>PH300</td>
<td>Computer</td>
<td>USB</td>
<td>USB</td>
</tr>
<tr>
<td>PDM SPAD #1</td>
<td>Timing out</td>
<td>PHR 800</td>
<td>Ch 1</td>
<td>LEMO</td>
</tr>
<tr>
<td>PDM SPAD #2</td>
<td>Timing out</td>
<td>PHR 800</td>
<td>Ch 2</td>
<td>LEMO</td>
</tr>
<tr>
<td>PDM SPAD #3</td>
<td>Timing out</td>
<td>PHR 800</td>
<td>Ch 3</td>
<td>LEMO</td>
</tr>
<tr>
<td>PDM SPAD #1</td>
<td>TTL out</td>
<td>Flex031a-01</td>
<td>A</td>
<td>BNC</td>
</tr>
<tr>
<td>PDM SPAD #2</td>
<td>TTL out</td>
<td>Flex031a-01</td>
<td>B</td>
<td>BNC</td>
</tr>
<tr>
<td>PDM SPAD #3</td>
<td>TTL out</td>
<td>Flex031a-01</td>
<td>C</td>
<td>BNC</td>
</tr>
<tr>
<td>Flex031a-01</td>
<td>USB</td>
<td>Computer</td>
<td>USB</td>
<td>USB</td>
</tr>
</tbody>
</table>
Emission is directed through the microscope to the right-hand side port, where it enters the photodetector module (Figure 29). A 50 µm pinhole mounted on a three-axis mechanical stage is positioned at the port’s focal point and reduces the focal depth of detection. The emission beam is then collimated by a singlet lens and split by a dichroic beamsplitter with 555 nm cutoff on a magnetic mount. The transmitted beam is focused by a singlet lens to one of three single-photonic avalanche photodiodes (SPADs, PDM-series, Optoelectric Components, Kirkland, QC, Canada) on a three-axis stage. The reflected beam was directed to a polarizing beamsplitter cube (Newport 10FC16PB.3) and similarly focused at two other SPADs.

**Instrument connections**

Single-photon streams are collected in time-tagged, time-correlated (T3) mode using a PicoHarp 300 time-correlated single-photon counting (TCSPC) system. Pulse synchronization requires electronic connections between illumination, pulse-picking, and TCSPC components (Figure 30 and Table 3). Because typical fluorophores exhibit lifetimes on the order of three nanoseconds, TCSPC measurements at full 80 MHz pulse frequency would misallocate a large share of photon counts from earlier pulses in the 12.5 ns data collection range. For this reason, a pulse-picking scheme reduces the effective OPO pulse frequency to 10 or 20 MHz.

The Ti:Sapph laser pulses at 80 MHz, and provides synchronization outputs corresponding to 40 MHz unrectified mode-locker and 80 MHz internal photodiode signals. The 40 MHz output is connected to trigger a ConOptics 305 countdown module, which reduces the output frequency by a factor of \( N + 1 \), where \( N \) is set by a dial on its panel. The unit’s “analog” output is connected directly to the amplifier of a ConOptics Model 25D Pockel’s cell, which modulates the tunable laser beam when voltage bias is properly configured. The ConOptics 305 “sync” output slaves the PicoQuant PDL-800-B diode laser via one of two sub-nanosecond delay modules (DB64, Stanford Research Systems, Sunnyvale, CA). When configured to external triggering, the PicoQuant laser is synchronized with the tunable laser pulse, and the delay between pulses can be optimized manually.

The PDL-800-B also triggers the “Ch0” pulse reference single for the PicoQuant TCSPC hardware. Although this can technically be achieved with any synchronized pulse, the pulse specification appears to be optimized between PicoQuant products for synchronization. For this reason, the PDL-800-B should be powered on even if the laser itself is not in use. This path incorporates an addition DB64 delay module to provide control over pulse collection within the TCSPC photon-counting window.

The three SPADs are designed to be powered by 12 V DC adapters, but instrumentation-grade DC power supplies have been attached to reduce possible cross-talk effects in two of the three detectors. SPADs provide both a low impedance output via a LEMO 00 connection and a conventional TTL output via a BNC cable. Because of the convenience of hardware FCCS, TTL outputs are connected to a Flex031q-01 hardware correlator (Correlator.com, China). Hardware correlator signal in the Flex software package also allow convenient real-time feedback of each SPAD signal during detector alignment. LEMO 00 connections link SPADs to the PicoHarp PHR 800 router, which multiplexes all three signals into a single TCSPC channel (Ch1) on the PH300 card.
**Hardware setup protocol**

The following procedures have been developed to configure the above fluorescence spectroscopy system for simultaneous TRFA and FCCS measurement.

**Microscope startup**

The MetaMorph control software frequently encounters errors if hardware components have been powered off. To avoid these problems, the following hardware startup sequence is advised.

1. Power on Sutter Instruments MOM-SCNDRV6/E scanner driver
2. Power on ASI MS-2000 stage
3. Power on Sutter Instruments LAMBDA 10-3
4. Power on Nikon Intensilight C-HGFI lamp power supply
5. Power on Nikon BF lamp controller
6. Restart Nikon scope
7. Power on Uniblitz shutter driver VMM-D3
8. Set filter cubes: RICM in position #3, laser notch filter in position #4
9. Restart acquisition computer
10. Install dichroic below microscope stage, tighten with Allen wrench

**Laser startup**

All configurations begin with startup of the Inspire OPO. If desired, the internal beam can then be diverted from the OPO cavity and instead operate in SHG mode, allowing access to 400-500 nm by tuning the IR wavelength of the MaiTai laser. For FCCS of GFP and mCherry fluorescent proteins, the PicoQuant diode laser provides 488 nm excitation, and the OPO remains at its default 550 nm startup wavelength.

1. Power on Conoptics Model 305 countdown module
2. Confirm connectivity between MaiTai mode lock, Pockel’s cell, and diode laser (diagram)
3. Turn on switch in back of PDL 800-B diode laser, set trigger to "external" (uses sync even if not lasing)
4. If using PDL 800-B diode laser: turn key, set to maximum intensity
5. Confirm that entry shutter to Inspire OPO is closed (clockwise position)
6. Turn key to SpectraPhysics power supply to "On"
7. Start "MaiTai Control" software on laptop
8. Use default COM port (COM port 8), press "OK"
9. Confirm that MaiTai starts up at 820 nm
10. Press and hold "On" for about 5 seconds, MaiTai software will take several minutes to complete warmup sequence
11. Once warmed up, press and hold "On" again; "Laser is on" will display and "Emission" warning will appear at top of screen
12. Wait until lasing begins, i.e. "IR Power" meter at right side of window rises to mid-range after several minutes.
13. Once IR power is about 2.6 and "Pulsing" is lit, click and hold "Shutter". MaiTai laser is now directed at OPO entrance.
15. Position hand above entry shutter to OPO. Open shutter at same time that spectrum plot appears in Inspire window.
16. OPO should home and identify peak of 550 nm.
17. If OPO has difficulty homing, confirm that OPO wavelength is 550 nm. Run "coarse cavity length" search.
18. Set OPO to desired wavelength or leave at 550 nm for SHG mode.
19. If SHG line is desired, enter SHG mode in Inspire software.
20. Wait for SHG line to home, confirm 410 nm emission from SHG port.
21. Set MaiTai wavelength as needed, e.g. 970 nm for 485 nm SHG emission.
22. Select "Find" in Inspire SHG window.
23. After SHG line homes, select "Optimize" and confirm desired wavelength at SHG port.

**Laser alignment**

This laser alignment procedure must be confirmed for each experiment. For safety, keep beams shuttered when manually aligning an optical component and wear protective eyewear where appropriate. Preserve cardboard beam enclosures as frequently as possible. Pockel’s cell body is highly reflective, so confirm beam position on paper to prevent hazardous reflections.

1. Close all OPO output shutters.
2. If using PicoQuant diode laser, confirm that PicoQuant beam enters and exits single-mode fiber.
3. If necessary, align PicoQuant laser using only steering mirrors that are specific to that line.
4. When PicoQuant laser is aligned, block laser head or turn off key for OPO alignment.
5. Start Pockel’s cell, set voltage to about -100 V so that cell transmits beam.
6. Confirm that OD1 neutral-density filter is between OPO output port and Pockel’s cell.
7. Move Pockel’s cell variable neutral density filter counterclockwise to limit maximum transmission limit.
8. Flip appropriate steering mirrors that direct line from OPO cavity or SHG output port to Pockel’s cell.
9. Cover Pockel’s cell entry with paper.
10. Open appropriate OPO output port.
11. Adjust steering mirrors so that laser beam overlaps Pockel’s cell entry aperture.
12. Project beam from Pockel’s cell exit onto card.
13. Adjust steering mirrors to shape first-order beam through Pockel’s cell.
14. Align beam to center of single-mode fiber entry aperture.
15. Fine-tune steering mirrors to maximize OPO/SHG transmission through fiber.
16. Confirm beam is centered on Uniblitz shutter.
17. Open UniBlitz shutter channel 3 to pass laser beam through back shutter.
18. Install linear polarizer, confirm proper angle and center beam
19. Measure laser power immediately after it exits single-mode fiber. Typically operating powers are 1-10 μW for each laser. Adjust as needed using variable ND filter at Pockel's cell for OPO/SHG line, and intensity control knob on PDL-800-B panel for diode laser.
20. Confirm that optical table is floating
21. Remove nosepiece cap and confirm laser beam is near spot on enclosure ceiling
22. If coarse adjustment is needed, position beam on ceiling using two actuators of bottom periscope mirror immediately after linear polarizer, and two axes of dichroic mirror using 1/16" balldriver
23. Return UniBlitz shutter #3 switch to software control
24. Mount objective lens and dispense NF immersion oil
25. Mount control sample, e.g. supported lipid bilayer or FITC solution
26. Confirm microscope body tube lens knob is set to 1x, not 1.5 x magnification
27. Focus on control sample using RICM and lock-in PFS focus. If using solution sample, focus at top of PFS range to bypass surface effects
28. Using "CRR_MaiTai_CCD" preset, align beam to center of viewing window (can use "show_crosshairs.jnl" to show spot). Confirm that beam does not saturate Andor CCD at 30 ms exposure.
29. If beam changes shape or position from center when slightly defocused, correct beam skew using same pairs of actuators as previous step. Confirm that beam remains circular and center across full trim range of PFS control.

**Detector alignment**

Excessive light can damage SPADs. Confirm that emission beam does not saturate Andor CCD at 30 ms exposure, as described in previous step. Keep room lights off and SPAD module in dark enclosure before activating SPAD power supplies

1. Confirm again that test sample fluorescent spot does not saturate Andor CCD at 30 ms exposure time.
2. Open Flex software and observe SPAD photon count rates, which should all equal zero before SPADs are powered on. Begin long (e.g. 3600 sec.) hardware correlator exposure.
3. Power on SPADs using DC power supplies and observe signals in Flex. Signals should rise to about 1 kHz for 10 seconds before falling to background count rate of 25-100 Hz. If at any point signals exceed 1000 kHz, immediately deactivate SPAD power supplies and troubleshoot source of illumination
4. Entire Metamorph Live mode with "CRR_MaiTai_FCS" present. Monitor SPAD signals in Flex.
5. If using SLB calibration sample, adjust PFS fine focus to maximize SPAD signals. If using solution sample, focus at lowest possible beam position at bottom of sample well.
6. Remove 50 μm pinhole by unscrewing tube. Maximize each SPAD's intensity by adjusting all three axes of its stage.
7. Re-insert pinhole. Make fine adjustments to pinhole position to further maximize power. Avoid adjusting axial pinhole position if using calibration solution, as this position needs to stay optimized for SLB samples.
Figure 31 – Power calibration for fluorescence spectroscopy. At laser powers beyond green dashed line, intensity response becomes nonlinear, indicative of saturation effects.

Figure 32 – Optimization of linear polarizer angle to achieve maximum anisotropy of reflected laser beam at photodetectors.
**Set pulse timing**

1. Start PicoHarp software
2. Configure constant fraction discriminator (CFD) voltages to register all SPAD signals
3. Set PicoHarp to about 5 second integration mode, with automatic restart.
4. Begin live TCSPC view and confirm that fluorescence decays are visible.
5. Block PicoQuant diode laser beam, if in use.
6. Rotate monitor to be visible from rear of optical table. Fluorescence peaks should be the same amplitude and separated by 12.5 ns in TCSPC when Pockel’s cell voltage is 0 V.
7. Adjust Pockel’s cell voltage to maximize picking modulation of a single peak, typically ±70-80 V. Peak should be as bright as possible, but signal before peak should also be very low. This is to eliminate fluorescence emission from molecules that were excited by earlier excitation pulses.
8. For single-color TRFA, the two polarization-split SPADS simply need to be positioned in the TCSPC time range. Adjust front DB64 delay box accordingly. The signal rise corresponding to the laser pulse should occur slightly after 0 ns, and the tail should be minimally truncated by the end of the range.
9. If also using PicoQuant diode laser, unblock beam and block OPO output port. Position pulse in TCSPC window, using both rear and front DB64 delay boxes.
10. Unshutter both beams and confirm that both diode laser and OPO pulses are appropriately delayed. For FCCS, pulses should be separated for pulse-interleaved excitation, i.e. decay of one fluorophore should not be detected in the others’ emission detection window.
11. Enter TTTR mode in PicoHarp software and record test PT3 file. Exit TTTR mode and confirm that pulse timing remains correct in PT3 file.

**Standard fluorescence experiment**

1. Load solution calibration standard, e.g. FITC or AlexaFluor dye in water. Focus PFS at top of range.
2. Record PT3 file. Load and confirm that polarization-split signals show anisotropy effect of mobile dye. Parallel SPAD signal should show visibly faster decay than perpendicular signal in first 0.5 ns following laser pulse. Alternatively, solution calibration can be loaded in PRADEEP and anisotropy decay confirmed.
3. After calibration, do not adjust photodetector or pinhole alignment. If this becomes necessary, another calibration measurement must be recorded.
4. Stop Live mode, load bilayer sample, focus PFS, and verify that signal does not saturate Andor CCD.
5. Start Live mode with "CRR_MaiTai_FCS" setting.
6. Optional: confirm that photobleaching does not occur by recording total APD response signal for various laser powers (Figure 31). Adjust variable neutral density filter so that experiment is performed with laser power corresponding to linear fluorescence response, safely below the onset of saturation and photobleaching effects.
7. Record file in TTTR mode.
As needed: calibrate linear polarizer position and measure instrument response

The linear polarizer angle should be aligned to either the reflected or transmitted orientation of the polarizing beam splitter cube in the photodetector module. When measuring directly back-reflected laser light, this will maximize the observed anisotropy in the polarization-split SPAD signals. However, exceptional caution needs to be used during this procedure, as the excitation laser beam exceeds the SPAD damage threshold by many orders of magnitude. This requires high attenuation of the OPO/SHG beam using both fixed and variable neutral density filters, as well as multiple confirmations of laser intensity at the Andor CCD before directing light the SPADs. The same optical configuration can be used to measure the dynamics of instrument response. Recorded instrument response functions (IRF) are later reconvolved during anisotropy and lifetime fitting to improve the accuracy of fitting rapidly decaying signals.

1. Set OPO line to 550 nm as above
2. Confirm neutral density filters to minimum OD1 in OPO path
3. Turn variable ND filter in Pockel's cell to absolute lowest setting
4. Remove laser blocking filter from position #4
5. Metamorph: Live mode with "CRR_MaiTai_CCD" setting to direct to Andor CCD
6. Confirm that exposure is 30 ms in Live mode
7. Increase variable ND filter at Pockel's cell until spot appears and is safely below saturation in CCD
8. End Live mode
9. Power on APDs and begin monitoring signal in Flex software, confirming presence of dark counts < 0.1 kHz
10. Block right-hand microscope side port with index card
11. Start Metamorph Live mode with "CRR_MaiTai_FCS" setting
12. While monitoring APD signals in Flex, quickly move card out and back into side port path
13. If any APD signal is more than 100 kHz during this procedure, stop Live mode, further reduce intensity at variable ND filter, then repeat
14. When stable APD power of 50-100 kHz is achieved, adjust linear polarizer angle in 5 degree increments and record both polarized APD signals (Figure 32)
15. Set LP angle to maximize anisotropy as close to 1 as possible
16. Record IRF until 10 million or more total counts are registered in TTTR mode

Software for anisotropy fitting

Time-tagged files are imported into PRADEEP, a software package written in MATLAB with additional functionality compiled from C. Menu options are available to import photon-counting stream files with PicoQuant T3R and PT3 extensions, and to save MATLAB system state to the workspace. Intensity and anisotropy signals on the order of seconds are displayed in left-hand plot. Central plots report TCSPC data for the entire file, and right-hand plots can be assigned to specific time ranges (regions of interest) in a given file. Bottom center and bottom right plots are assignable for TRFA, lifetime, and FCCS modes. Key parameters to process and fit TRFA decays are described here.
Display and fit ranges

Minimum and maximum times of displayed TCSPC data are assignable in nanoseconds. Similar fields define the start and finish of curve fitting in TRFA and lifetime modes. In general, fitting range needs to be optimized for a given experiment. Fitting should start at the illumination laser pulse and end shortly before the next pulse arrives, often an attenuated peak that is not perfectly suppressed during pulse picking. Because of instrument response dynamics, fitting of slower anisotropy decays will need to begin at slightly later times. Hence, the start of fitting should be slightly later in proteins than calibration solutions.

“Flip” setting

The detectors parallel and perpendicular to laser polarization may be assigned to either router channel. By default, ChA is parallel, but setting “flip” will reverse this routing in software analysis.

Detector calibration factor

A calibration solution of small organic dye should be recorded and imported into PRADEEP. To set the $G$-factor, anisotropy decay should be analyzed with appropriate fit ranges, and the model set to single-exponential decay to a constant (“exp1wt_c”). Starting with $G = 1$, increase or decrease $G$ until the constant “r_inf” becomes as close to zero as possible. If hardware is properly configured and mechanically stable, this calibration factor remains valid until detectors are realigned.

Instrument response function reconvolution

In general, TRFA decays are affected by the response dynamics of photodetectors and other electronics. To compensate for this, it is standard practice to convolve the empirically measured IRF with lifetime and anisotropy fit models. For a new IRF recorded using the previously describe procedure, import the T3R or PT3 file, and export as a MAT file in PRADEEP using the “Export as IRF” menu option. This file can then be loaded and reconvolved automatically by selecting “Load IRF”.

Binning

PicoQuant TCSPC systems allow resolution to be varied by powers of two, from a minimum of 4 ps on PicoHarp and 32 ps on TimeHarp. High TCPSC resolution describes rapid anisotropy events, such as those associated with small organic rotational diffusion or homo-FRET. However, high resolution also spreads the same number of photons across a larger number of bins, increasing the variance of photon counts in a single bin and reducing the validity of Gaussian distribution inside each channel. In extreme cases at the tail of anisotropy fits, integer rounding effects are visible. In many experiments, it is desirable to identify fast anisotropy decay events as well as slow rotational diffusion, and hence apply analysis at both resolutions. By setting the TCSPC capture to low resolution, however, information about shorter events is lost. To facilitate both high and low resolution analyses, a “rebin” feature has been included to repeat curve fitting at both high and low resolution.
In reality, maximum TCSPC resolution is not feasible for many experiments, because the number of captured bins is conserved. Using the lowest bin width of 4 ps in PicoHarp reduces the collection window to 12.5 ns, hence truncating the tails of fluorescence data.

**Relative delay**

Channels can be delayed by small amounts < 200 ps due to differences in optical path length in detector module. Longer delays are also possible in some configurations due to different standards of LEMO cable that connect the photodetectors and TCSPC router. To correct this, a relative delay adjustment is included. Using the calibration solution measurement in anisotropy mode, adjust relative channel delay until TCSPC signal rise rates are coincident during the laser pulse. This parameter should not require adjustment as long as the electronic and optical configuration remains unchanged. By erroneously varying this delay, it is clear that fast anisotropy artifacts can be induced.

**TCSPC signal delay**

Collected photons are sometimes far from the earliest bins in the TimeHarp TCSPC card. Because this TCSPC data collection is a periodic cycle, it is also possible for collected photon histograms to start late in the collection window and decay with tails that reset to the beginning of the window. This adjustment simply shifts both detector signals by a fixed amount.

In the PicoHarp 300 model, the number of assignable bins is far higher. Hardware delays are set to position the collected photon histograms early in the collection window, so adjustments to this setting are rarely needed.

**Regions of interest (ROI)**

Specific time ranges within a photon-counting experiment can be defined and computed as subsets in TRFA, lifetime, and FCCS analysis. ROI can be defined by specific time intervals, or by intensity above a given threshold as measured by sequential point measurements that are interrupted by shuddering the detector channel.

**Regression for anisotropy fitting**

Several methods have historically been developed to fit the anisotropy decay laws to the constitutive photodetector signals in Eq. 7. Most methods, however, require additional insight into the overall intensity decay law independent of polarization. This can be obtained by independent magic-angle polarization measurements or sum intensity normalization as performed in lifetime analysis\(^\text{11}\). However, both means of independently establishing the sum intensity signal are complicated by the high-NA optics of this system. In general, the method developed herein needed to be robust to a variety of probes and conditions, and derived from identical photon-counting files without intermittent reconfiguration of the optical system. The added complexity of simultaneous fitting was therefore judged unacceptable, and the imprecision of rapid depolarization events tolerated, for these protein diffusion experiments.

Nonlinear least squares (NLLS) fitting was used to minimize the goodness of fit statistic defined by Eq. 29. The residual weighting function was defined from propagation of Poisson photon errors as described in Eq. 28, separately reconvolving the two TCSPC intensity channels with their respective IRFs. Levenberg-Marquardt optimization was then
performed using the MATLAB nlinf( ) function. In the case of lifetime fitting, single intensity traces are fit independently with the same function, using appropriately modified Poisson variance weighting.

**High numerical aperture correction**

The linear polarization field implicit in the definition of anisotropy in Eq. 5 is only strictly attainable using collimated optics. In systems where excitation, detection, or both fields are manipulated by focusing, electric field distributions are no longer homogenous and their component normal to the plane of polarization must be figured into anisotropy\(^{129,140–142}\). Higher deviations from the paraxial approximation lead to higher distortions in anisotropy; this effect increases with numerical aperture in the specific instance of confocal fluorescence optics\(^{143}\). Adjustments for this effect on fluorescence anisotropy were first studied in the context of widefield imaging of membrane probe orientation in polarization imaging of erythrocytes\(^{144}\), whereby a simple transform between laboratory and sample coordinates relates observed with actual anisotropies. In PRADEEP analysis software for TRFA, this correction is reduced to a NA-dependent scalar correction factor, which scales the peak anisotropy to near its ideal maximum of 0.4 in calibration solutions.

More complicated corrections based on spherical coordinates have been developed specifically for high numerical aperture confocal systems for which inhomogeneities in point-spread function and alignment of polarization elements may affect determination of absolute anisotropy, including extension to evanescent-field excitation as observed in total internal reflection (TIRF) microscopy\(^{140–142}\). As these effects are often difficult to precisely measure in a real fluorescence system, Koshioka and colleagues\(^{145}\) simplified the Cartesian transforms to two parameters that can then be calibrated using a sample of known single-exponential anisotropy decay. However, experimental studies have demonstrated that only the magnitude of anisotropy, and not its time dynamics, are distorted by this optical effect\(^{122}\).

When depolarization is on the same timescale as fluorescence decay, measurements of fluorescence lifetime can also be effected by anisotropy effects. In a paraxial system, this is corrected using either magic angle polarization or “total intensity” averaging of components parallel and perpendicular to polarization of illumination. In high numerical aperture systems, however, these corrections are similarly affected\(^{141,142,146}\).
Correlation analysis for FCS and FCCS

Calculation of correlation and cross-correlation functions can be achieved using the "multi-tau" method based on a common hardware correlation routine\textsuperscript{147}. However, this forces a Poisson process into synchronous data bins, compromising either data resolution or requiring massive system resources to correlate. To improve the speed and accuracy of fitting, an algorithm conceptually outlined in a prior publication\textsuperscript{148} was adapted for integrated FCS and FCCS analysis in PRADEEP (Figure 33).

TTTR data import scripts \textit{mex\_import\_t3r.c} and \textit{mex\_import\_pt3.c} were adapted from scripts supplied with PicoHarp software. Functionality was added for time gating and ROI ranges, and single-photon streams are written to two MATLAB vectors. This script was originally developed to attain \(N\) from single-species FCS to compare anisotropy with local surface concentration. Since then, the script has been applied in Ras and lipid anchors projects to infer actual translational diffusion and cross-correlation from samples, respectively.

As \(\tau\) approaches the total time duration of a given measurement or ROI, correlation can rapidly fall in one or both channels. This has been partially improved by recognizing that the finite integration time \(\Delta t = t_f-t_0\) of a measurement effectively limits the upper limits of integration in the correlation calculation:

![Algorithm diagram](image-url)
Because the signal does not exist at low $t_f$, the upper limit of integration $t_1$ must be correspondingly reduced to $t_f - \tau$ to normalize for the limited range of data available at higher $\tau$. Although this effect does not appear in several other correlation scripts or using hardware correlation, this may be because the corresponding algorithms estimate fluctuations around random ($G(\tau) = 1$) correlations.

Despite drastic improvements in computation time for large T3R/PT3 files, batch processing of experiments can still take several hours. This complicates a key feature of PRADEEP, namely the ability to rapidly view correlation data from many experiments in a batch. This has been partially alleviated by the “pre-compute” option in PRADEEP, by which all open ROI are processed and correlation results saved in dynamic memory. Further improvements in FCCS function fitting can be obtained by weighting residuals with an appropriate signal variance$^{147}$. 

\[
G(\tau) = \frac{\frac{1}{t_1-t_0} \int_{t_0}^{t_1} A(t)B(t+\tau)dt}{\frac{1}{(t_1-t_0)\tau} \int_{t_0}^{t_1} A(t)dt \int_{t_0}^{t_1} B(t)dt}
\]

Eq. 39
System characterization

Spot size calibration

Beam waist size is typically calibrated using a known concentration of small organic fluorophore, and comparing its diffusion time in FCS with its known diffusivity at room temperature. FCS of quickly diffusing species generally requires cross-correlation between two channels, because the fluorescence signal is lost in afterpulsing effects on the sub microsecond timescale. Spot size was calibrated with a 100 nM solution of fluorescein isothiocyanate (FITC) at room temperature, diluted with water from 500 nM stock in a 400 µL NUNC plate well. Perhaps due to additional timing artifacts from the PicoHarp TCSPC electronics or limitations to the masHyper() algorithm, this sample’s cross-correlation function is clearer using hardware correlation than software (Figure 34).

Fluorescein diffusion coefficient was reported as 422-437 µm$^2$ s$^{-1}$ at 22-23 °C in literature measurements$^{149}$, and can be fixed during FCS curve fitting to calculate beam
waist and length dimensions. One potential limitation of this method is the potential for large variations in dye diffusivities with preparation and experiment temperature. Small organic fluorophores in solution were used to establish the calibration $G$-factor, and were chosen to match the fluorescence absorption and emission bands of probes used in main experiments. Both FITC (Figure 35A) and AlexaFluor 594 (Figure 35B) show rapid decays on the order of 100 ps. When $G$ is properly set, anisotropy decays to a steady state of zero. Rotational correlations of purified His10-GFP in solution are reported in the next chapter. Treatment with antibody against GFP predictably slowed the anisotropy decay, approximately doubling the protein’s RCT in solution.

**Instrument response functions**

IRFs measured using the above method show a very rapid pulse rise, followed by a somewhat slower multiexponential with an initial decay constant of about 100-200 ps (Figure 37). Two photodetectors’ IRFs were very nearly identical, and were positioned to measure both polarization-split channels. The third photodetectors’ decay was somewhat different, and did not change with signal routing or power supply. When used in TRFA, this discrepancy caused a slight decay artifact at the 100 ps timescale (not pictured).

**Homo-FRET**

Homo-transfer between adjacent fluorophores is known to cause a rapid decay in fluorescence anisotropy at the scale of 100 ps. Although this has been characterized with steady-state anisotropy in samples that are essential immobile, the presence of rotational diffusion generally also depolarizes fluorescence, necessitating time-resolved measurements to ascertain the presence or absence of homo-FRET in a sample. Similarly, nonradiative energy transfer between adjacent fluorophores may limit the brightness of clustered molecules, requiring measurements of fluorescence lifetimes at this timescale in

![Figure 36](image-url)
order to validate the assumption that fluorescence brightness is not influenced by self-interactions. These two effects are described in more detail in Chap. 4.

Several controls confirm this system’s ability to observe homo-FRET and self-quenching at these timescales. First, the system features sufficient time resolution to measure anisotropy decays near 100 ps, as evidenced by clear resolution of time decays in small organic calibration solutions (Figure 35). Secondly, samples of GFP began to exhibit short anisotropy decay events after many months of refrigerator storage, consistent with gradual protein aggregation with interfluorophore distances in the range for Förster interactions. Finally, dedicated GFP-GFP fusion proteins showed similar fast anisotropy decays imposed on slower components attributed to rotational diffusion (not pictured).

**Conclusions**

Rotational diffusion in membranes has been reviewed, and a theoretical model has been developed to describe the recovery of rotational correlation times using time-resolved fluorescence anisotropy. Hardware and software have been developed to perform TRFA in a confocal microscope using time-correlated single photon counting. Measurement protocols and system performance have been described, enabling simultaneous measurements using anisotropy, correlation, and lifetime spectroscopy. This provides a versatile photonic platform with which to study membrane diffusion and its relation to viscous microenvironments and intermolecular interactions. In subsequent chapters, the utility of this system is demonstrated on a number of protein systems in vivo and in vitro.

This fluorescence spectroscopy system can be further developed to enable anisotropy imaging, which has already been demonstrated for the simple case of time-independent anisotropy\textsuperscript{152}. Rastered imaging of time-resolved responses can theoretically produce fully resolved time decays at each pixel, although this requires either high laser intensities or short exposure times. Laser powers are potentially limited by probe photobleaching and TCSPC pile-up effects. Shorter exposures result in lower pixel photon counts and highly non-Gaussian count distributions in each TCSPC channel. A fitting method such as maximum likelihood estimation (MLE) would be required to sensibly fit sparser anisotropy time traces\textsuperscript{153}, or else simplified decay estimation algorithms could instead be adapted from fluorescence lifetime imaging (FLIM) to determine the rate of anisotropy decay\textsuperscript{126,154}. These challenges are, however, solvable with current technology. Extending this fluorescence spectroscopy system to full-frame imaging would augment a powerful analytical tool for a diversity of biophysical problems.
5. TRFA OF POLYHISTIDINE-ATTACHED PROTEINS ON BILAYERS

Introduction

The fluorescence spectroscopy platform described in the previous chapter is capable of simultaneously characterizing the rotational and translation diffusion of proteins on supported lipid bilayers using TRFA and FCS, respectively. As SLBs are increasingly used to reconstitute complicated membrane signaling pathways, there is a need to characterize the mechanical behavior of SLB-tethered proteins and its dependence on attachment chemistry. This chapter describes the characterization of proteins attached to SLBs using chelation of polyhistidine tags, with a specific emphasis on intercellular adhesion molecule-1 (ICAM-1), plays a vital role in the T-cell immunological synapse. This incorporates material from the following two manuscripts:


and


Motivation

The cell membrane is a crucial signaling hub that concentrates and organizes diverse proteins. How the two-dimensional membrane environment alters the equilibrium and kinetics of protein-protein interactions has been modeled for decades. Reconstituted model membrane systems, notably supported lipid bilayers (SLBs), can isolate membrane protein functions from thousands of other participants to assist spectroscopic characterization. The chelation of polyhistidine to metal-coordinated lipid headgroups has been a particularly useful tool with which to study spatially organized structures in cells, notably the T-cell costimulatory function of intracellular adhesion molecule-1 (ICAM-1) alongside antigen-peptide major histocompatibility complex. Although this strategy offers precise control over protein stoichiometry on SLBs, the mechanical properties of polyhistidine-lipid attachment are largely uncharacterized.

Rotational diffusion of membrane-associated molecules can be accurately predicted from a simple hydrodynamic model, and is therefore an attractive indicator of local viscous environment and intermolecular interactions. Time-resolved fluorescence anisotropy (TRFA) has provided insight into the rotational diffusion of small molecules and peptides in solution, as well as membrane protein clustering via resonant energy transfer. However, protein rotational diffusion is slow compared to the nanosecond-order lifetime of fluorescence emission. In time-resolved experiments, most photons are therefore captured before anisotropy has decayed appreciably, necessitating high
Illumination intensities or long integration times. For proteins on cells or vesicles, the available reservoir of fluorophores is rapidly depleted due to photobleaching, limiting the number of data points available to quantify anisotropy decay rates.

In contrast, SLBs are extensive, homogeneous, and fluid, so fluorescence from large ensembles of bilayer-bound protein can be integrated while rejecting background emission from bulk solution. Photon statistics from these large samples are then sufficiently large to study anisotropy decays exceeding ten fluorescence lifetimes.

**His10-GFP rotational diffusion: solution and membrane measurements**

To characterize the rotational diffusion of polyhistidine-linked protein on SLBs, a confocal optical system based on time-correlated single-photon counting (TCSPC) was focused at the SLB surface. Rotational diffusion was measured via time-resolved fluorescence anisotropy of polarized emission at two avalanche photodiodes and calibrated against known small organic fluorophores in solution.
Supported lipid bilayers were formed on #1.5 coverglass or glass-bottomed 96-well plates (NUNC, Inc.) etched with either piranha (3:1 H2SO4:H2O2) or sodium hydroxide, respectively. Lipid vesicles containing 2% 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid)succinyl] (DGS-NTA, Avanti Polar Lipids, Alabaster, AL) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti) were extruded and ruptured on glass substrates to form SLBs according to published protocols\textsuperscript{168}. Polyhistidine-tagged green fluorescent protein (His10-GFP) was attached by nickel salt chelation to a DOPC SLB containing 2% DGS-NTA (Figure 37A), allowing protein to diffuse across the optical detection volume without photobleaching.

Fluorescence anisotropy of His10-GFP exhibited anisotropy decay with an exponential time constant of 28.7 ± 2.2 ns (Figure 37B). His10-GFP from the same preparation showed substantially shorter exponential decay (18.6 ± 0.6 ns) when measured in bulk solution in the absence of a lipid bilayer (Figure 37C).

**Viscous model of protein tethering to membrane**

Beyond the simple hydrodynamic solution drag on His10-GFP, tethering and boundary effects may explain the observed 53% increase in RCT when attached to a SLB.

**Hydrodynamic contributions**

The Stokes drag factor of a freely tumbling sphere in solution is related to its RCT by Eq. 16. This yields a drag coefficient $f_{\text{soln}} = 75.8$ pN·nm·ns and an effective spherical radius $R = 2.6$ nm for His10-GFP in aqueous buffer at 22 °C. This differs slightly from a report of 2.4 nm in the literature\textsuperscript{108}, possibly due to the addition of active histidine residues as well as differences in protein preparation, and buffer temperature and viscosity. The calculated size is bounded by GFP “β-can” radius 1.5 nm and height of 3.0 nm reported from crystallographic studies\textsuperscript{169}.

An extensive theoretical field has been developed on the translational and rotational diffusivity of inclusions in supported lipid bilayers\textsuperscript{95,96,98,170}, but minimal theoretical insight
exists into the effect on diffusion of membrane tethering such as this His-tag strategy. The anisotropy optical path includes minor contributions from emission dipoles oriented outside of the plane of the bilayer, but these only affect the absolute anisotropy and do not substantially alter the measured decay rate\textsuperscript{144,145}. Hence, 2d rotational diffusion about the bilayer normal axis is a valid model of how solvent drag and lipid viscosity influence the His10-GFP rotational diffusion on bilayers.

**Tethering effects**

Tethering effects may also contribute to the RCT, because polyhistidine attachment increases His10-GFP’s effective gyration radius. In addition to friction drag from free diffusion in solution (Figure 38A), His10-GFP is also subject to the added drag of translational motion about its polyhistidine tether and its coupling to lipid diffusion. These effects are additive with the Einstein-Smoluchowski friction drag factor from $f_{\text{total}} = 117$ pN\(\cdot\)nm\(\cdot\)ns from Eq. 20:

$$f_{\text{total}} = f_{\text{soln}} + f_{\text{tether}} + \alpha f_{\text{lipid}}$$  \hspace{1cm} \text{Eq. 40}

where $\alpha$ is a lipid coupling coefficient. Friction factors $f_{\text{soln}} = 75.8$ pN\(\cdot\)nm\(\cdot\)ns and $f_{\text{lipid}} = 88.4$ pN\(\cdot\)nm\(\cdot\)ns are known using Eq. 16 and Eq. 20 from measured rotational correlation times.

Polyhistidine anchors the center of GFP random rotational motion to a point on the bilayer surface, and this impedes the random reorientation of its dipole about its center of mass (Figure 38B). The Einstein-Smoluchowski relation equates frictional drag effects in driven and random rotation. Hence the role of the His-tag tether can be quantified from an analogous Stokes flow experiment using a magnetically trapped bead and rod complex attached to surface-bound DNA\textsuperscript{171}. The rod offsets the bead’s axis of rotation from that of the DNA by length $L$, requiring that resistance to the circular translation motion be added to the friction drag coefficient in Eq. SI-7:

$$f_{\text{tether}} = \eta \pi RL^2$$  \hspace{1cm} \text{Eq. 41}

The drag of the peptide linker itself is neglected, so an additional term accounting for Stokes flow around the nanorod is omitted from this interpretation, as are coefficients that account for boundary effects near the static wall. The length $L$ between the centroid of GFP and its point of attachment depends on the conformation of polyhistidine, but can be approximated by a rigid $\beta$-sheet structure that extends 3.5 Å per histidine residue. The tether length is then approximately

$$L = 2.6 + 0.35x \text{ nm}$$  \hspace{1cm} \text{Eq. 42}

where the NTA-His bond is formed at the $x$ residue, and the 2.6 nm GFP Stokes radius accounts for the distance from the tag to the protein center of mass. If tethering always occurs at the most distant histidine site, $<L^2> = 37.2$ nm\(^2\) and $f_{\text{tether}} = 0.30$ pN\(\cdot\)nm\(\cdot\)ns, only 0.7\% of the difference $f_{\text{total}} - f_{\text{soln}}$. More realistically, a single NTA-His bond is equally probable at all ten sites, so $<L^2> = 21.5$ nm\(^2\) and $f_{\text{tether}} = 0.17$ pN\(\cdot\)nm\(\cdot\)ns. Even across the
maximum possible His-tag length, these tethering effects account for only 0.7% of the observed increase in RCT.

Hence, the additional drag caused by tethering His10-GFP to a freely mobile surface is insufficient to explain the observed change in rotational correlation.

**Diffusion near wall**

The amplification of a continuous fluid's viscous effects near solid boundaries is well known from boundary layer theory in fluid mechanics\(^{172}\). Hydrodynamic theory has been applied to the similar situation of a diffusing sphere near a solid wall, but results are qualitatively different for the rotational diffusion about the surface normal\(^{173,174}\). Faxen's laws relate surface stresses to an arbitrary viscous flow field\(^{175}\) and have been used to model sphere-sphere interactions\(^{176,177}\). Analogous to boundary layer theory, translational mobilities approach zero as a sphere contacts a wall, along vectors both normal to\(^{178}\) and in the plane of the surface\(^{179}\). Rotational mobility about the wall normal is reduced but does not approach zero at wall contact\(^{173}\). The reduction in mobility has been calculated numerically to be only 17% asymptotically close to contact\(^{174}\), and is therefore insufficient to explain the increase in His10-GFP RCT upon binding to bilayers.

**Lipid coupling in polyhistidine rotational diffusion**

Neither tethering nor boundary effects explain the increase in SLB-bound His10-GFP RCT, suggesting that His10-GFP diffusion is viscously coupled to the underlying bilayer. To quantify the degree of coupling across the polyhistidine linker, anisotropy decay of 0.01% Texas Red 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt (Texas Red DHPE, Invitrogen, Carlsbad, CA) (TR-DHPE) was measured in similarly prepared SLBs.

Texas Red is attached to the hydrophilic headgroup of DHPE and has been demonstrated to exist at a shallow bilayer depth and hence higher exposure to solution in both computational\(^{180,181}\) and experimental studies\(^{182}\). This suggests that TR-DHPE will exhibit two distinct anisotropy decay components: fast rotational diffusion of the exposed dye subunit, and slower diffusion of the whole lipid molecule. Indeed, TR-DHPE anisotropy decays were visibly two-exponential (Figure 37D). In contrast to His10-GFP measurements, goodness-of-fit statistics and randomness of fit residuals were both improved by fitting TR-DHPE anisotropy decay to a two-exponential function (Figure 39), yielding anisotropy decay time constants of 1.2 ± 0.4 ns and 21.7 ± 2.7 ns.
Because the TR-DHPE fast anisotropy decay component (1.2 ns) is on the same timescale as similarly sized FITC and AlexaFluor 549 small organic calibration dyes in solution, we conclude that this term describes exposed Texas Red subunit diffusion. The slower time component (21.7 ns) better agrees with a two-dimensional Stokes approximation of lipid rotational diffusion in a bilayer, and is therefore attributed to the slower rotational diffusion of whole lipid molecules in the bilayer.

By so separating the anisotropy decay of the Texas Red probe from that of its lipid molecule, the TR-DHPE slow rotational component can then be safely attributed to stronger viscous contribution from acyl chain diffusion. The question remains as to whether DHPE diffusion is representative of the contribution of DGS-NTA to His-tagged protein diffusion. Simulations have suggested that Texas Red may transiently dimerize adjacent phospholipid molecules, an effect that is manifested in slowed diffusion. So these measurements could slightly understate the rotational diffusivity of DGS-NTA, but this would not affect the conclusion that His-tagged protein is determined by lipid and solvent contributions in parallel.
If each polyhistidine tag rigidly binds a single Ni-DGS lipid molecule, Eq. 40 predicts that the RCT of bilayer-bound His10-GFP is simply the sum of free monomeric and lipid diffusion terms. However, the discrepancy between solution and bilayer-bound His10-GFP is only balanced when coupling to lipid diffusion is considered with coefficient $\alpha = 0.46$. In this model, $\alpha = 0$ corresponds to complete isolation of His10-GFP diffusion from bilayer viscosity, and $\alpha = 1$ if the protein is rigidly bound by a single histidine. An intermediate value of $\alpha$ indicates that partial coupling to lipid bilayer diffusion occurs, either by hindered rotational diffusion internal to the protein-lipid structure or time-averaged fluctuations between loosely and rigidly bound states. Considering the multivalency of NTA-DGS coordination bonds and the relative rigidity of the short polyhistidine linker, a possible source of hindered diffusion is that of the DGS lipid headgroup relative its hydrophobic tail.

**Lipid coupling factor to polyhistidine**

Figure 40 – (A) Time-resolved fluorescence anisotropy decay and single-exponential fit of His10-ICAM-YFP on SLB. (B) Treated with anti-GFP, (C) Treated with anti-ICAM, (D) Fluorescence correlation spectroscopy of His10-YFP-ICAM bilayer before (yellow circles) and after (red diamonds) incubation with anti-GFP. (E) Fluorescence correlation spectroscopy of His10-YFP-ICAM bilayer before (yellow circles) and after (red diamonds) incubation with anti-ICAM-1.
ICAM bilayers

After establishing that protein-lipid rotation is partially decoupled at the site of polyhistidine linkage, we investigated whether a similar effect can also occur internal to the structure of membrane proteins. A His-tagged fusion of yellow fluorescent protein and intercellular adhesion molecule-1 (His10-YFP-ICAM-1) similarly attached to SLBs decayed with RCT of $26.9 \pm 2.3$ ns (Figure 40A). Domain-specific crosslinking was then performed using antibodies specific to either YFP or ICAM-1. Monoconal antibody against GFP (Cell Signaling Technology, Danvers, MA), known to show high affinity for YFP within this same protein fusion, drastically slowed the decay of fluorescence anisotropy to $147 \pm 54$ ns (Figure 40B). His10-YFP-ICAM bilayers incubated with antibody against ICAM-1 (clone YN1/1.7.4, Abcam, Cambridge, MA) showed no significant change in RCT (Figure 40C).

Fluorescence correlation spectroscopy (FCS) was performed by auto- and cross-correlation analysis of the same TCSPC photon streams, and was used to compute the mean number of fluorescent protein molecules in the illumination spot. Molecular brightness, the ratio of fluorescence intensity to number of diffusing species, increased by $33 \pm 7\%$ when anti-GFP was added (Figure 40D). Molecular brightness in samples treated with anti-ICAM-1 increased by $99 \pm 30\%$, confirming that the antibody crosslinked most protein on the bilayer (Figure 40E).

Crosslinking His10-YFP-ICAM with anti-GFP considerably slows its RCT, and anisotropy decay is barely discernible within the span of the TCSPC trace. This is expected from the high sensitivity of in-plane lipid membrane rotational diffusion to the lateral dimension of a diffusing complex, which may exceed 10 nm based on the distance between antibody binding sites. Furthermore, RCTs in excess of 10 $\mu$s have been reported using time-resolved phosphorescence anisotropy of similarly crosslinked cell-surface receptors. Hence the measured RCT of this diffusing complex represents only a coarse estimate of its drastically slower rotational diffusion, and may include contributions from monomeric His10-GFP-ICAM on the bilayer. In sharp contrast to protein crosslinked with anti-GFP, anti-ICAM does not affect rotational diffusion despite a molecular brightness increase commensurate with nearly complete crosslinking. This indicates that substantial rotational flexibility exists between the YFP and N-terminus of ICAM-1, a region that spans five disulfide-bridged ICAM-1 active loops.

Figure 41 – Brightfield (left) and epifluorescence (right) images of His10-ICAM-YFP on SLB assembled in T-cell immunological synapse.
Anisotropy of ICAM-1 in T-cell immunological synapse

To explore rotational diffusion in vivo in a reconstituted membrane system, His10-YFP-ICAM-1 was attached to a SLB along with peptide-loaded major histocompatibility complex I (pMHC). As predicted, T-cells rapidly organized ICAM into a distinctive peripheral supramolecular adhesion cluster (pSMAC) (Figure 41), consistent with its organization via its LFA-1 cognate receptor. Despite dramatic accumulation of His10-YFP-ICAM at the bilayer interface, however, the anisotropy decayed from $r_0 = 0.30 \pm 0.02$ at rates that did not differ significantly between the ring (24.6 ± 2.8 ns) and bilayer measurements taken far away from cells (22.9 ± 1.6 ns). Large-scale ICAM clustering does not influence YFP anisotropy decay, confirming that substantial rotational flexibility exists within the His10-YFP-ICAM fusion protein.

Translational mobility and brightness in pSMAC

Several unpublished fluorescence experiments suggest that gradients in ICAM-1 mobility in the pSMAC are associated with oligomerization of LFA-1, its conjugate receptor in T-cells. In general, point observations of His10-YFP-ICAM1 emission in the pSMAC show rapid photobleaching to a steady-state intensity. The presence of such a photobleached fraction demonstrates that mobility is inhibited in this microenvironment. The slow fraction is highest near the center of the cell and decrease monotonically toward the periphery, despite the disruption of monotonicity in the distinctive pSMAC ring. Speckle tracking shows that the centripetal velocity of actin labeled with fluorescent protein is inversely related to slow fraction of ICAM-1, suggesting that this effect is dynamically linked to the cytoskeleton. Similar trends in molecular brightness were measured with FCS and photon-counting histogram (PCH) analysis, revealing that 35-37% of ICAM is dimerized in the pSMAC. These data suggest that an active sorting mechanism is present across the entire interacting surface of synapsing antigen-presenting cells.

However, these result could alternatively be explained by energy transfer between adjacent YFP molecules, either as radiative homo-FRET or nonradiative self-quenching. Homo-FRET, as described in the previous chapter, can be identified by its signature.

Figure 42 – Fluorescence lifetime analysis for both polarization-separated channels.
anisotropy decay at timescales of 100 ps. This event was completely absent in repeated TRFA measurements of pSMACs (Figure 41), so homo-FRET was readily ruled out.

Self-quenching classically manifests itself as a saturation in emission intensity with increasing valency of fluorophore labeling on proteins\textsuperscript{187,188}. In endogenously labeled molecules such as His10-YFP-ICAM-1, similar energy transfer mechanisms could be present due to close association of clustered fluorescent domains. Self-quenching is readily observable as a decrease in the nanosecond lifetime of fluorescence for the same excitation intensity, consistent with a decreased proportion of radiative decay from the fluorophore excited state.

Lifetimes of TCSPC measurements performed in both TRFA polarization channels (Figure 42) showed only a very small difference between ICAM-1 in the pSMAC (2.55 ± 0.02 ns) and that measured far away from cell synapses (2.62 ± 0.03 ns). In pSMACs that photobleached, the unbleached fraction did not show a significantly different lifetime (2.55 ± 0.03 ns). As described previously, differences in anisotropy decays were insignificant and hence did not influence lifetime measurements. Upon crosslinking with anti-GFP, His10-YFP-ICAM-1 bilayers showed only a small change in lifetime (from 2.67 ± 0.01 ns to 2.64 ± 0.01 ns) despite a dramatic anisotropy change (Figure 40B). However, crosslinked ICAM did show a decrease in lifetime (to 2.48 ± 0.04 ns) in pSMACs after photobleaching, suggesting that perturbation of intrinsic ICAM clustering alters the photophysics of YFP emission in this environment.

**Discussion**

Conformational flexibility in His10-YFP-ICAM-1 similarly decouples the T-cell membrane from viscous effects in the SLB, potentially biasing receptor-ligand interactions in which a cell’s mechanical environment is interpreted as a biochemical signal\textsuperscript{189}. The presence of such interdomain flexibility also has important implications for the role of ICAM-1 in cell adhesion and costimulation. Rhinovirus neutralization with ICAM-1-immunoglobulin fusions is known to depend on the number of ICAM-1 domains as well as linker flexibility, suggesting that the dense attachment of this ligand in the viral capsid is aided by its low torsional rigidity\textsuperscript{190}.

Because of this flexibility, polarization effects do not influence uncorrected comparisons of fluorescence lifetime between ICAM-1 in the pSMAC and the surrounding bilayer. These measurements confirm that translation diffusion and brightness exhibit a spatial gradient through the synapsing interface, and provide insight into cytoskeleton-linked trafficking mechanism in T-cell membranes.

**Conclusions**

Supported lipid bilayers integrate a continuously diffusing fluorescent sample to achieve high precision of proteins’ slow rotational diffusion. The rotational correlation time of His10-GFP increased when attached to the bilayer, an effect that is consistent with partial coupling to the lipid bilayer. Domain-specific crosslinking of His10-YFP-ICAM-1 shows that ICAM-1 exhibits substantial rotational flexibility, hence decoupling its cell-interacting region from potential viscous effects in the model membrane system. Despite
clear trends in the organization of ICAM-1 across its interface with T-cells, rotational diffusion was not affected. Together, these results show that engineering a model bilayer’s viscosity is not sufficient to mimic proteins’ mechanical environment in physiological membranes. As the utilization of reconstituted membrane systems grows in popularity, often in conjunction with sophisticated protein fusions, such sources of rotational flexibility must be considered in systems where mechanical feedback is suspected.
6. FLUORESCENCE SPECTROSCOPY BELOW THE RESOLUTION LIMIT

Introduction

Experiments described in the previous chapter used fluorescence anisotropy to address impediments to diffusion posed by a protein’s environment. This occurred in clearly resolvable accumulations at the same size scale as cells, and rotational diffusion was characterized as an internal property of the His10-YFP-ICAM-1 fusion molecule. The following experiments were contributions to separate projects in the laboratory in which anisotropy was combined with other fluorescence spectroscopy methods to study interactions at substantially scales, and hence variations were not resolvable in fluorescent images.

Ras dimerization

Background

Previous experiments in the laboratory confirmed the presence of an allosteric Ras binding site on SOS that greatly increased its catalytic turnover when bound on supported lipid bilayers. Single-molecule assays also showed distinct fluctuations in the rate of Ras turnover, suggesting a source of structural heterogeneity that in turn affected the reaction kinetics. However, experiments were complicated by a number of changes in the physical behavior of Ras bilayers, including translational mobilities that appeared to vary with surface concentration and illumination conditions in FCS (unreported). TRFA was therefore employed to further investigate the physics behind these observations.

Ras shows two-component anisotropy decay

Previously reported mutants of wild-type H-Ras(C181,C118S) (residues 1–181, C118S) and mutant H-Ras(Y64A, C118S) were tethered to MCC-DOPE supported lipid bilayers via coupling between the Cys181 residue and maleimide on lipid molecules. Time-tagged single-photon streams of fluorescence emission Atto488-labeled nucleotide (GDP or GTP) were recorded using the method described in Chaps. 3-4, and processed for TRFA and FCS analysis with 3 μW power from the Inspire SHG line tuned to 485 nm. Surface density of Ras was measured to be 935 μm⁻² using software FCS of the same photon data.

Because the Atto488 fluorophore is linked to Ras via a dynamically bound nucleotide, multicomponent rotation was strongly suspected. Both wild-type and Y64A variants (henceforth referred to as WT- and Y64A-Ras) were indeed better fit by two-exponential anisotropy decay models (Figure 43A) than by a single-exponential decay (Figure 43B) based on the randomness of fit residuals as well as the relative sum squared residual ($\chi^2_r$). Fast exponential decay components on the order of 1 ns agreed with the anisotropy decay rate of both labeled nucleotides in solution (Figure 43C-D). The fast component was therefore attributed to hindered subunit rotational diffusion of the nucleotide when bound to Ras. Slow components of WT-Ras (12.7± 3.2 ns) and Ras-Y64A (9.3 ± 0.6 ns) were then concluded to describe the rotational correlation of Ras itself, and referred to as the RCT of these two Ras species.
Figure 43 – Wild-type and Y64A Ras are both better fit by two-exponential anisotropy decay (A) than single-exponential model (B), based on central tendency of residual and sum of square residuals (not pictured). Fast decay components are $0.79 \pm 0.33$ ns and $0.76 \pm 0.15$ ns, respectively, and agree closely with both labeled GDP (C) and GTP (D) anisotropy decays in solution. Slow anisotropy decay of WT-Ras ($12.7 \pm 3.2$ ns) and Ras-Y64A ($9.3 \pm 0.6$ ns) are therefore attributed to protein rotational correlation.
WT-Ras shows slower RCT than Y64A-Ras

To investigate the possible dependence of Ras mobility on photoexcitation power, anisotropy of WT-Ras and Y64A-Ras bilayers was measured across a range of laser powers. Nucleotide TRFA components (Figure 44A) showed neither a significant difference nor a clear dependence on laser power. Slow components attributed to Ras rotational diffusion, however, were significantly higher for WT-Ras than for the Y64A mutant across the entire intensity range (Figure 44B). Dependence on laser intensity, however, was quite weak and accounted for a far smaller difference in RCT across this range. This was concluded to belong to natural photophysical artifacts such as TCSPC pileup effects.

Because these two Ras variants do not differ greatly in size, differences in RCT suggested that a structural difference was present. This result could be explained by two possibilities. First, WT-Ras may oligomerize, increasing the effective gyration radius of the complex on the bilayer and hence slowing RCT. Alternatively, WT-Ras may associate differently with the membrane, in this case attaching to an additional lipid site that similarly slows its rotational diffusion.

WT-Ras RCT increases with concentration

To distinguish these two possibilities, further comparisons of Ras RCT at different surface concentrations were conducted. As measured by FCS, a variety of WT-Ras surface densities were obtained by varying loading concentration in flow cells. Substrates prepared in this way were also known to show a factor of 2-3 gradient in concentration due to irregularities in their flow fields, so further variation in surface concentration was obtained by measuring at multiple locations, separated by 2-5 mm, in a single sample. For TRFA measurements, error bars were obtained from 10 independent 15-30 sec. measurements separated by about 10-50 μm.
Two-color FCS of Ras-Atto488 and TR-DHPE was performed on a separate system, resulting in a comparison of Ras lateral mobility normalized for the local bilayer fluidity (Figure 45A). Consistent with earlier observations, WT-Ras diffused more slowly at higher concentrations, while Y64A-Ras mobility stayed the same relative to TR-DHPE. The effect was the same regardless of whether GTP or GDP was used. A similar trend in TRFA was observed for WT-Ras (Figure 45B). WT-Ras RCT at surface densities from 33 to 160 µm⁻² were measured in a single day, and an additional data point from an earlier experiment at 935 µm⁻² (Figure 43A) was also included. Together, these experiments show a logarithmic increase in RCT with surface density.
Similar TRFA characterizations of Y64A-Ras were also attempted, but due to mechanical alignment problems in the photodetector system, normalization $G$-factors drifted severely. This was indicated by fluctuations in $r_0$ that occurred while measuring Y64A-Ras samples. TRFA estimates were obtained by floating $G$ and fixing $r_0$ at a similar value to WT-Ras measurements, although this method resulted in much larger variations in each data point, disguising any functiona dependence on surface concentration that may have been present.

Considering the hypothesize structural explanations for WT-Ras mobility and RCT trends, Y64A was expected to maintain a roughly constant RCT that was faster than WT-Ras with increasing concentration similar to these FCS observations. Although the RCT of Y64A was noticeably shorter in later measurements, only the earlier Y64A measurement at high concentration ($9.3 \pm 0.6$ ns at $935 \text{ µm}^{-2}$) could be reported with confidence. Indeed, this RCT is slower than that of low concentrations of WT-Ras. However, this was not wholly unexpected because these experiments were separated by six months and may have featured substantially different bilayer properties. Additionally, crowding effects that become more common at higher concentrations may indeed increase Y64A-Ras RCT somewhat. Nonetheless, TRFA characterization of these two Ras mutants agree with FCS observations.

Crucially, photon-counting histogram (PCH) analysis demonstrates WT-Ras dimerization with quantifiable dissociation constants (Figure 45B). This method quantifies the molecular brightness of Ras, which would be expected to increase if oligomerization occurs. However, energy transfer between fluorophores may also change molecular brightness, necessitating time-resolved characterization of fluorescence lifetime to confirm that PCH does not describe photophysical differences in the two fluorescent nucleotides.

**Self-quenching and homo-transfer interactions**

Fluorescence lifetime analysis was performed to evaluate whether self-quenching or homo-transfer interactions could explain decreasing WT-Ras rotational and translation mobilities at high concentration, as well as brightness effects observed in PCH. As introduced in the last chapter, both radiative and nonradiative energy transfer may occur between fluorophores separated by subnanometer distances. Nonradiative self-quenching effects may hence decrease a sample’s quantum efficiency with increasing concentration on a membrane, manifested as a decreasing fluorescence lifetime\(^{191}\). Ras-bound Atto488-GTP and –GDP both exhibited two-component fluorescence lifetimes in time-resolved measurements at magic-angle polarization that minimizes anisotropy effects. Although previously unreported, the highly-charged nucleotide may contribute an additional charge transfer pathway that contributes substantially to radiative excited state decay in Atto488. However, the fluorescence lifetime components of Ras did not change significantly with concentration or in the Y64 mutant, verifying that the observed brightness results are not influenced by photophysical interactions between like fluorophores.

Radiative transfer in the form of homo-FRET is generally manifested as rapid depolarization with time constant less than 1 ns in time-resolved fluorescence anisotropy\(^{123,125}\). Anisotropy measurements of both wild-type and mutated Ras bilayers already exhibit rapid anisotropy decay at this timescale (Figure 43C-D) attributed to
rotational diffusion of the fluorescent nucleotide relative to Ras protein. The presence of an additional anisotropy decay component that is obfuscated by this fast decay cannot be ruled out. However, this fast component does not differ significantly between wild-type and mutant Ras or with increasing wild-type concentration. Furthermore, concentration-dependent trends in Ras rotational correlations occur at a substantially slower time scale than that expected for homo-FRET.

**Conclusions**

Energy transfer between fluorophores does not contribute to the observed trends in diffusion and brightness. Although both translational and rotational diffusion trends could be explained by either increased WT-Ras oligomerization or protein-lipid binding, the PCH results confirm that the first explanation is correct. Furthermore, PCH shows a distinct doubling of molecular brightness that, in the absence of confounding photophysical interactions, specifically demonstrates that the interaction is specifically dimerization. All three methods confirm that the Y64A mutation abrogates this dimerization.

**Single-molecule photophysics in immobilized vesicles**

**Motivation**

This platform for photon-counting fluorescence spectroscopy on supported lipid bilayers typically averages a large ensemble of molecules as they diffuse through the confocal optical volume. Although the mean number of molecules in the observation
volume can be diluted to one or fewer, FCS demonstrates that in highly fluid SLBs molecules spend 100 ms or less in the observation volume.

Single-molecule characterization of membrane protein function is desirable for a number of reasons. Most importantly, relating fluctuations in structural and functional variables would present an extremely powerful platform to characterize the physical basis of enzymatic reactions. Functional readouts are readily available in a number of fluorescent systems, notably the gradual loss of fluorescence in fluorescent nucleotide-Ras bilayers due to exchange of unlabeled nucleotide by SOS (unpublished).

In the context of fluorescence, a dynamic means of characterizing structural variations in an enzyme is more difficult. This requires site-specific attachment of fluorescent molecules and, in the case of single-molecule studies, the ability to immobilize the protein of interest while still preserving its ability to function. Site-specific labeling of proteins is possible using a variety of chemistries, including highly photostable small organic dyes that are favorable for single-molecule measurements. Site-specific fluorophore pairs can be generally engineered with sufficient precision to signal structural variation using FRET. Although this is generally averaged across large ensembles, single-molecule FRET is possible and has provided insight into the specific receptor-ligand signaling in T-cell immunological synapse.

**Method and Preliminary Results**

Single Atto647N-SOS molecules were encapsulated in BODIPY-labeled SUVs and immobilized to glass coverslips using poly(L)lysine – poly(ethylene glycol) copolymer using previously reported methods. Single SUV-encapsulated SOS molecules were identified in two-color TIRF imaging and coarsely positioned using the microscope’s mechanical stage. The confocal fluorescence spectroscopy beam was slowly rastered in a 6 x 6 grid with step sizes of 100-500 nm, dwelling 2-30 seconds on individual pixels while continuously collecting polarization-split photon signals in TCSPC. Following TCPSC measurements, the presence of single molecules was confirmed by continuing TIRF exposure until the Atto647N channel photobleached, an event which was confirmed to occur with a single step (Figure 46A).

Coincidence of the optical focus and single SOS molecules was clearly observed via a sharp spike in photodetector intensity, as observed in real-time hardware correlator measurements. In post-processing of TCSPC photon streams, background noise that occurred outside of these emission windows could be reduced by limiting the time range of analysis. TCSPC intensity decays showed clearly resolvable fluorescence lifetimes in both polarization channels (Figure 46B), and anisotropy decreased at nanosecond timescales (not pictured). Furthermore, lifetimes were still recoverable after dividing the TCSPC data into two-second ROI. Changes in intensity correlated with lifetime fluctuations, and this was further supported by Bayesian inference of intensity signals (Figure 46C). Although further study is required, this suggests that dynamic heterogeneity in single protein emission was observed, perhaps due to fluctuating proximity between the fluorophore and inner surface of the SUV.
Conclusions

This chapter has demonstrated additional functionality of the previously described fluorescence spectroscopy platform. In particular, time-resolved anisotropy measurements provide structural insight that would not otherwise be possible with time-independent or correlation methods. In Ras, anisotropy contributions from protein oligomerization were distinguished from the hindered diffusion of labeled nucleotide. In single-molecule studies of vesicle-encapsulated SOS, the continuous integration of TCSPC showed clearly resolvable fluctuations in probe photophysics. By combining anisotropy with simultaneous FCS and lifetime characterization, this platform’s ability to measure structural variations will continue to be an asset to studies of Ras-SOS and other membrane protein projects.
7. FUTURE DIRECTIONS

While the techniques described in previous chapters will remain applicable to a broad spectrum of biophysical questions, perhaps no application is more exciting than the pursuit of single-molecule protein measurements. In the context of laboratory-scale biological assays, single-molecule protein methods have revolutionized the scope of discovery in the past ten years. These experiments have provided unprecedented insight into the enzymes that cells use to move, convert chemical energy, and communicate with their host organisms. Accounting for molecular diffusion, optical localization, and the photophysics of fluorescence emission have all been crucial dimensions of this rapid period of growth in single-molecule research.

Whereas single-molecule information addresses the intrinsic heterogeneity of protein structure and function in biological research, it offers a fundamentally different benefit for environmental biosensing. For applications in security and public health, many of the advantages of laboratory assays are absent: the distance between sensor and specimen cannot be reduced to millimeters or less, target molecules cannot be quickly concentrated in a centrifuge, and diagnostics decisions must be made in seconds or less. For this reason, sensitivity is the most critical performance specification, and engineering sensor systems for single-molecule detection should be the primary goal for fieldable devices. Owing in large part to transport limitations, the detection of a single molecule can be the difference between a detected and ignored threat.

Fortunately, the themes of engineering surface diffusion, nano-optics, and spectroscopy are all highly relevant to sensor development, and form the basis of a sensing strategy that I now propose.

Surface diffusion

Recent flow physics research has addressed transport limitations in mass-adsorption biosensors\textsuperscript{195,196}. A recognition probe, such as an antibody, aggressively binds its target molecule in these devices. Performance is therefore severely rate-limited by diffusion: a \(1\mu m\) sensor detects the first molecule of a \(1\) fM solution after an average of one hour\textsuperscript{195}. The specifications for environmental sensing only exacerbate this problem: the mean detection time can often be days or weeks. Although preconcentration can partially accelerate this response, the same physical effect depletes the local concentration of the target molecule\textsuperscript{196} and hence severely limits sensitivity.

This challenge raises an important materials science question: can a surface selectively entrap a molecule while preserving its lateral mobility? In fact, supported lipid bilayers already exhibit a similar behavior, and are frequently used to mimic the surfaces of cells. Using reactions such as polyhistidine chelation, proteins from liquid solution can be attached to lipid surfaces with high mobility\textsuperscript{197} that interact readily with photonic nanostructures\textsuperscript{165}. Although these lipid membranes are unstable outside of aqueous solution, similar surface entrapment properties can be achieved by embedding recognition probes in thin lubrication layers of oil or synthetic polymer. Hence, nanofabrication methods to partition and assemble supported lipid bilayers are highly relevant to a surface entrapment strategy that can maximizes the utilization of single adsorbed analyte molecules.
Photonic sensing is perhaps the most attractive platform for high-sensitivity signal transduction. In contrast to surface acoustic-wave (SAW) and nanowire field-effect transistor (NW-FET) devices, optical probes are frequently bound directly to recognition biomolecules, providing a dimension of control that is not possible in other systems. Furthermore, inexpensive light sources and detectors are now ubiquitous in mobile electronics and continue to excel in performance and complexity.

Recent scientific advances in nano-optics offer enormous potential for biosensing, but substantial engineering is required to reliably obtain single-molecule sensitivity\textsuperscript{13}. Dominant label-free biosensing signal transduction schemes such as localized surface plasmon resonance (LSPR) and surface-enhanced Raman spectroscopy (SERS) rely on integration of scattering spectra from hundreds of single detection events. Label-free single-molecule surface-plasmon sensing has only been demonstrated in carefully controlled laboratory conditions\textsuperscript{198}. However, energy transfer to nanometallic surfaces can selectively boost or suppress probe emission, and this offers a pathway for single-molecule detection.

Although electron resonance in metals can either enhance or quench a probe’s emission, the physics of the latter process have barely been explored for sensing\textsuperscript{8}. Several probes are excellent candidates for this design: metal nanoparticles and Raman probes scatter light, and quantum dots and synthetic chromophores emit red-shifted fluorescence. Nanostructures fabricated using electron-beam lithography (EBL) and physical vapor deposition (PVD) can be tested against a variety of probes to maximize the modulation of photonic signal to noise. Further insight into the photophysics of this background-suppression system can quantified by measuring photon emission rates using time-correlated single photon counting (TCSPC), providing a means of optimizing photoquenching to maximize signal-to-noise for single detected molecules.

\textit{Irreversible probe reactions}

Antibodies purified from the immune system exhibit strong affinity for foreign molecules, and can be engineered against a broad variety of targets. For this reason, antibody capture is the dominant functionalization strategy in biosensors for diagnostic...
applications. Although providing exceptional selectivity, antibody simply immobilizes its antigen without changing state at the timescale needed for biosensing. For this application, recognition probes are needed that:

1. Meet or exceed the selectivity of antibodies in order to minimize false-positive signals
2. Irreversibly change state when a target molecule is identified
3. Can be engineered to recognize a broad variety of threats, and
4. Share common chemical motifs that can be used for surface attachment

Fortunately, rapid advances in nucleic acid aptamers meet these conditions. Using in vitro evolution techniques, aptamers have been engineered to identify a broad array of targets with high selectivity\textsuperscript{199}. Aptamer “beacons” that couple a fluorescent molecule with a complementary quenching molecule are the most attractive solution: when the aptamer recognizes its target, the fluorophore separates from the quencher and the beacon is free to emit a fluorescent signal (Figure 48A-B).

Beacons are generally reversible, meaning the emission signal disappears once the target molecule unbinds. Irreversible fluorophore interactions, however, are feasible and will be pursued using several published biochemical strategies. In one variation, hybridization of probe sequences competes with binding of the target molecule, releasing fluorescent signal in solution when the target is identified\textsuperscript{200} (Figure 48C). Another possible strategy is the incorporation of restriction endonucleases, enzymes that slice DNA at specifically encoded locations, to permanently liberate the fluorophore and quencher upon target recognition\textsuperscript{201} (Figure 48D).

Importantly, this strategy will use metallic nanostructures, instead of chemical quenchers, to suppress probe recognition if the target molecule has not been identified (Figure 48B-D). One additional advantage of nucleic acid-based recognition chemistry is the ability to pattern microarrays into multiplex assays\textsuperscript{202}. These two features will allow integration of this biosensing platform into larger arrays against a variety of targets, allowing many parallel biomarkers to be screened.
Conclusion

During my doctoral research, I have engineered nanofabricated materials and time-resolved fluorescence spectroscopy to address the unique analytical challenges of protein detection at membrane interfaces. Nanofabricated plasmonic structures shrink light to the size scale of protein interactions. Similar fabrication technology, when adapted to the unique surface chemistry needs of supported lipid bilayers, can provide exceptional control over protein diffusion and achieve single-molecule sensitivity in vesicle assays. Fluorescence spectroscopy has provided insight into mechanical flexibility between and within biomacromolecules, and these variables have been used to infer important interactions in signaling systems. Together, these experimental methods have measured and controlled diffusion at nanometer length and nanosecond time scales.

Future directions for basic research have been introduced throughout this dissertation. Nano-optical structures may be able to further shrink the accessible size scale of fluorescence measurements, past the scale of single molecules to structural fluctuations between domains of the same molecule. Time-resolved fluorescence detection can be extended with scanning imaging to simultaneously perform multiple spectroscopic characterizations in entire cells. The same techniques can be combined with diffusion models for a general platform to characterize protein oligomerization across a broad range of chemical conditions, providing insight into such nanoscale organization in live cells.

But as an engineer, the opportunity to extend these techniques from the laboratory to practical applications is perhaps the most exciting direction. Biosensors can be in the hands of medical experts, first responders, and military personnel to rapidly identify and intercept biological threats from disease outbreak and bioterrorism. Nanotechnology-based approaches to single-molecule sensing will be beneficial for point-of-care medical diagnostics and environmental monitoring. Crucially, the role of protein structure and organization amplifies the importance of this research for sensing and diagnostic applications.

Just as motion sensing, imaging, and voice recognition have become ubiquitous consumer technology, I aspire to bring a precise understanding of biological signals to society’s toolbox.


