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Advancements in Single Molecule Localization Microscopy for Improved
Multichannel, 3-Dimensional, and Structurally-Dense Imaging

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A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2019

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Abstract

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Super-resolution fluorescence microscopy now enables cellular detail to be probed at the nanometer scale using far-field optics with widely available instrumentation. However, single molecule localization microscopy (SMLM) techniques that rely on the stochastic on-off switching of individual fluorophores to achieve higher resolution face significant drawbacks when trying to achieve multicolor, 3-D, and structurally-dense imaging. This is due to limitations in the photoswitchable fluorophores currently available. Here, I provide a detailed discussion of photoswitchable fluorophores for SMLM and the challenges they create. I also present a new method, prSTORM, which is capable of using a single fluorophore to image all channels and achieve 3-D imaging at extended depths through the use of DNA barcodes. Additionally, I propose how this methodology can be extended to high structural density imaging without compromising resolution.

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ACKNOWLEDGEMENTS

Far more has happened in the past six years than I ever could have anticipated when embarking on this journey. As a young student coming from a small liberal arts and science college no one quite prepared me for exactly what I was getting into when entering graduate school. Sure, I knew there would be classes and research, but not fully what that meant. For in getting to this point, I have gained much more than another degree. I have experienced highs and lows both professionally and personally throughout my time here and without the solid support system of people around me I would not be the person I am today.

My decision to choose UW for graduate school was largely based on two reasons. The first of which was visitation weekend happened to be one of the lucky beautiful sunny weekends in March we sometimes get. With the vibrant colors of campus springing to life and Mount Rainer in view along Rainer Vista, who could not fall in love with the place? The second was because of a person I met at one of the dinners, who at first glance I thought to be another prospective student. He later introduced himself as Joshua Vaughan, a new faculty higher. While we did not speak for long, or very much about research, our conversation left me with a very good feeling about UW and the prospect of having him as an advisor. After committing to UW I was fortunate to have him agree to take me on as a summer student and then as a graduate student. I could fill pages with all the details of what I am grateful to Josh for over the years, from taking the initial chance on me as one of his first graduate students, to always having his door open to hear professional and sometimes personal matters. I thank him for being a kind and understanding person every step of the way, constructive but not too critical, patient, and so much more. I am grateful I chose to join his lab six years ago and that he chose to let me.

During my time in the Vaughan Lab I have had the privilege of working closely with two great scientists on my main research projects, Danying Lin and Marco Howard. As a visiting scholar for only a year, Danying's time with us was far too short. However in that year I gained so much knowledge from her mentorship and grew substantially as a scientist. I believe the greatest lesson I will remember from her is to "Just be happy" and that is an attitude I hope to carry with in my career and life. Marco, I am glad I hesitantly allowed Josh to put you on my project all those years ago. It was great always having someone to bounce ideas off of, you have been a great lab partner

and I will miss working with you. I am not really sure how you will function when I leave but I know you will find a way. At least now you have less keys so it won't take you so long to open the office door...hopefully.

I was fortunate to join a lab where the atmosphere feels a lot like family, much of that has to do with the members who joined early on, Tyler Chozinski, Aaron Halpern, Min Yen Lee and also Marco. Tyler was there since day one and I am thankful that he quickly became a good friend. His kindness is limitless, I recall him not getting remotely angry at me when I accidentally spilt a bucket of iced water on his feet while outside on a cold October night during our stint as Graduate Club Social Chairs. Aaron has been an integral part of the lab since he joined a few months after Tyler and I with what seems like an infinite amount of patience. He has always made himself available to troubleshoot experiments, lend a helping hand, and to talk all things cats and Great British Bake Off. Last, but certainly not least, Min Yen, who has always been a good sport and lifted other people's spirits up. Thank you for putting up with my shenanigans and being there for everyone. I thank the four of you tremendously for making this journey better and fun (sometimes).

I'd also like to acknowledge the support of all the other past and present members of the Vaughan Lab: Honglin Li, Hyeon-Jin Kim, Tran Lee, Ethan Vo, Sarah Parkhurst, Grant Tremel, Marcus Woodworth, Chenyi Mao, Jing-Ru Jhan, and Jonathan Perr. In addition, I would like to thank Professor Kai-Mei Fu and her graduate student, Maria Viitaniemi, of the Physics Department for welcoming me into your lab for several months while collaborating on an exploratory research project together.

Finally, I would like to thank my family, my parents and brothers for their continuous support throughout the years. Particularly my parents who have always believed in me and never allowed me the opportunity to doubt my own capabilities. Lastly, my new family, my husband Dima, who has literally been with me since day one of this process—we had our first date after my first official day of graduate school orientation. I feel somewhat like this PhD belongs a little bit to him as he has been there through every single high and low giving unwavering support, encouragement, and advice. I have been fortunate enough to have had a home I love coming back to each night and that has made a world of difference. I look forward to what the next chapter in our lives will bring.

CHAPTER 1. INTRODUCTION

1.1 THESIS STRUCTURE

This thesis is organized into 4 major chapters. The first includes a brief overview of super-resolution microscopy with greater emphasis on single molecule localization microscopy (SMLM), the focus of my research. Chapters 2-4 consist of major publications and protocols that I co-authored.

1.2 BRIEF OVERVIEW: OBSERVING A HIDDEN WORLD

There exists an entire world beyond the one we can touch and see with our naked eye. Figure 1.1 is an artistic rendition of a small region of a eukaryotic cell. It is highly organized and composed of many tiny building blocks that work together to accomplish a wide range of functions. Understanding how these parts are assembled and work together down to the nanometer scale is one of the grand challenges scientists across a wide range of disciplines face due to the small size and density of these parts.

Optical microscopes have given people the ability to access this hidden world and serve as a powerful tool for solving biological problems. Their development and improvement has often coincided with major scientific breakthroughs, from the discovery of key fundamental optical phenomenon (Airy discs, Abbe's diffraction limit, Köhler Illumination, etc.) to measuring the ~37 nm step size of the myosin V motor protein along actin filaments¹. Fluorescence microscopy in particular has become a workhorse tool for biologists and development of super-resolution microscopy has enabled many fundamental biological discoveries such as the periodicity of actin rings in neuronal axons²; characterization of the γ -complex in nuclear pore complexes³, and detection of interactions among single species⁴ or between multiple⁵. With further development these methods will continue to revolutionize the field of biology.

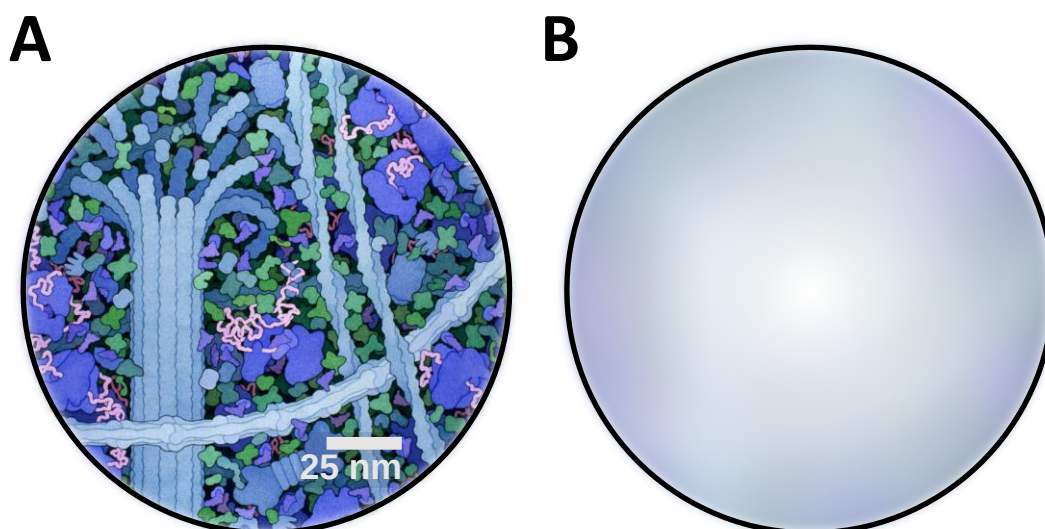


Figure 1.1. Biology beyond the Diffraction Limit. A) A small portion of cytoplasm is shown, including three types of filaments that make up the cytoskeleton: a microtubule (the largest), an intermediate filament (the knobby one) and two actin filaments (the smallest ones). The large blue molecules are ribosomes, busy in their task of synthesizing proteins. The large protein at bottom center is a proteasome. Illustration and caption used with permission by David S. Goodsell, the Scripps Research Institute. B) The level of detail observable within this region using traditional microscopy due to the diffraction limit.

1.2.1 *Light microscopy and the diffraction limit*

Basic light microscopes were introduced in the early 17th century and modern conventional light microscopes enable us to resolve objects separated by as little as ~200 nm. Fluorescence microscopy, which utilizes fluorescent molecules to label the sample, adds a degree of specificity to light microscopy. Rather than seeing everything that absorbs and scatters as you would with transmitted light, fluorescence microscopy allows you to observe only the sites bearing a bright fluorescent tag against a nearly zero background. While fluorescence has greatly enhanced the utility of light microscopy, its resolution is still limited to around 200 nm. This is due to the diffraction limit of light, otherwise known as Abbe's diffraction limit, which prevents resolving objects closer than roughly one-half the wavelength of visible light⁶. More specifically, Abbe's diffraction limit is defined by the equation $d \approx \lambda/2NA$, where d is spatial resolution, λ is the wavelength of light, and NA is the numerical aperture of the microscope objective lens.

1.2.2 *Alternatives to light microscopy*

Techniques like electron microscopy (EM) and atomic force microscopy (AFM) can obtain a spatial resolution well beneath the diffraction limit of visible light (~200 nm) even to the point of resolving individual atoms in a material. While EM has very high spatial resolution, it suffers from low contrast and poor ability to identify specific molecules being studied. AFM has similar drawbacks in addition to being limited mostly to a sample's surface.

1.3 BRIEF OVERVIEW OF SUPER-RESOLUTION MICROSCOPY TECHNIQUES

To surpass the diffraction limit of light and to maintain the high contrast and specificity of fluorescence microscopy, a variety of super-resolution microscopy techniques have been created in the last two decades. These methods now allow us to peer beneath blurry diffraction-limited images to better observe the fine details of cellular organization. In fact, these methods have already made such a strong impact on the field that two of them were the topic of the 2014 Nobel Prize in Chemistry⁷⁻⁹.

1.3.1 *Stimulated Emission Depletion Microscopy (STED)*

STED microscopy originated from the lab of Stefan Hell, one of the recipients of the 2014 Nobel Prize in chemistry⁸, and is similar in setup to a confocal microscope, where an excitation beam is focused down to a point on a diffraction limited sample and subsequently scanned over the entire volume. Before signal reaches the detector it passes through a pinhole which is used to block signal from out of focus emitters which are normally observed with widefield microscopy. This improves the quality of fluorescence images by reducing the background signal however there is a tradeoff when it comes to pinhole size. The pinhole also blocks some signal from in focus emitters and if the size of the aperture is too small then it can end up blocking too much meaningful signal to have a positive effect on signal-to-noise.

The key difference between confocal and STED microscopy is that STED microscopes use two illumination beams to create an illumination pattern which is scanned through the sample (Figure 1.2). The first beam is used to excite fluorophores labeling a specimen. The second, red-

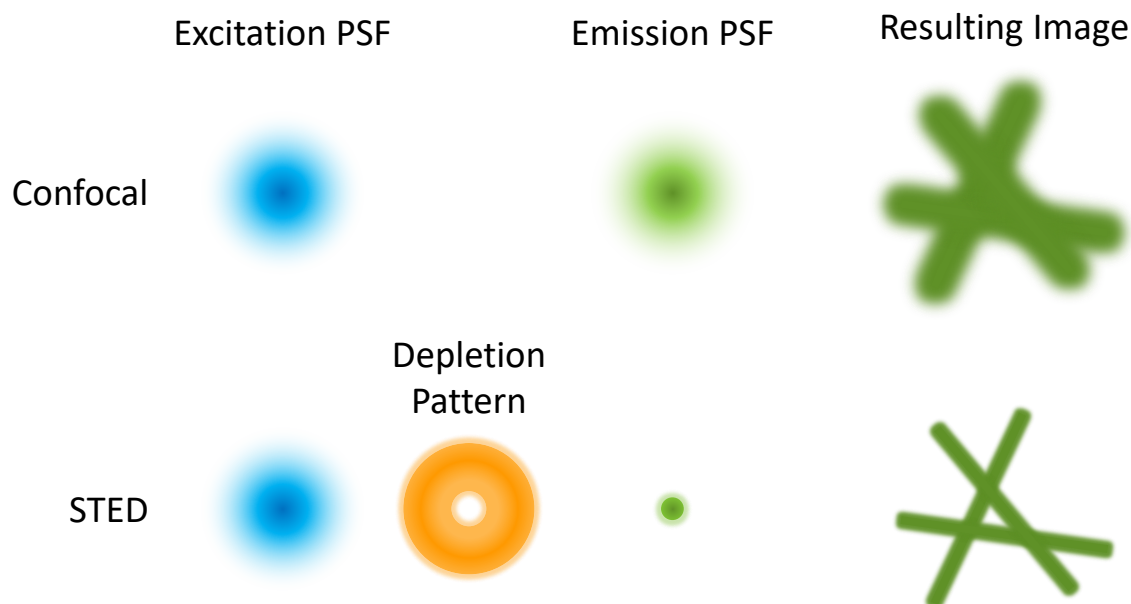


Figure 1.2. Comparison of confocal and STED microscopy. *Top row:* Illustrates the excitation and emission point spread functions (PSF) you see with confocal microscopy. The emission PSF matches the excitation PSF and results in a diffraction limited image. *Bottom row:* The excitation PSF is identical to that of a confocal microscope. A donut-like depletion pattern is overlaid on the excitation beam which results in a much smaller, not diffraction-limited, emission PSF. This allows a high resolution image to be collected, and fine details (triangular region in the middle) to be observed.

shifted, “depletion” beam is shaped using adaptive optics to form a donut-like ring with a zero-intensity feature at the center, meaning no light at that wavelength exists in the middle. The wavelength of the second beam is red-shifted from the excitation beam, such that any fluorophores in the excited state due to the first beam will immediately transition back to ground state via a process called stimulated emission when exposed to the second beam. The stimulated emitted photons exist at the same wavelength of the red-shifted beam making them spectrally distinct from excited molecules. The excited molecules fluoresce and their emission is able to be detected in a spectral band that is unique from both the excitation and depletion wavelengths. The donut beam is superimposed over the excitation beam such that only the very small hole of the donut remains in the excited state; this effectively narrows the diffraction-limited point spread function and routinely achieves a spatial resolution of 30-70 nm¹⁰⁻¹². It is important to note that STED requires the use of special fluorophores that are particularly resistant to photobleaching which will be discussed further in **Chapter 2**.

1.3.2 Structured Illumination Microscopy (SIM)

SIM is a second patterned illumination technique regularly capable of obtaining an approximately two-fold enhancement in spatial resolution. In the most common form of SIM, patterned illumination of a sample through the use of a grating or other beam shaping objects in the excitation path is used to generate interference patterns with the sample, also known as Moiré fringes. A series of images is recorded for several angles and phases of the illumination pattern and then the series of images is computationally analyzed to allow the reconstruction of a high resolution image (Figure 1.3). This is possible because the interaction between the high frequency objects (that are separated by less than the ~ 200 nm diffraction limit) within the sample and the high frequency illumination pattern generates a lower frequency pattern from which the high frequency spatial information can be extracted. This process can be configured to work in both two or three dimensions and routinely achieves a lateral spatial resolution of ~ 110 nm^{13–15}.

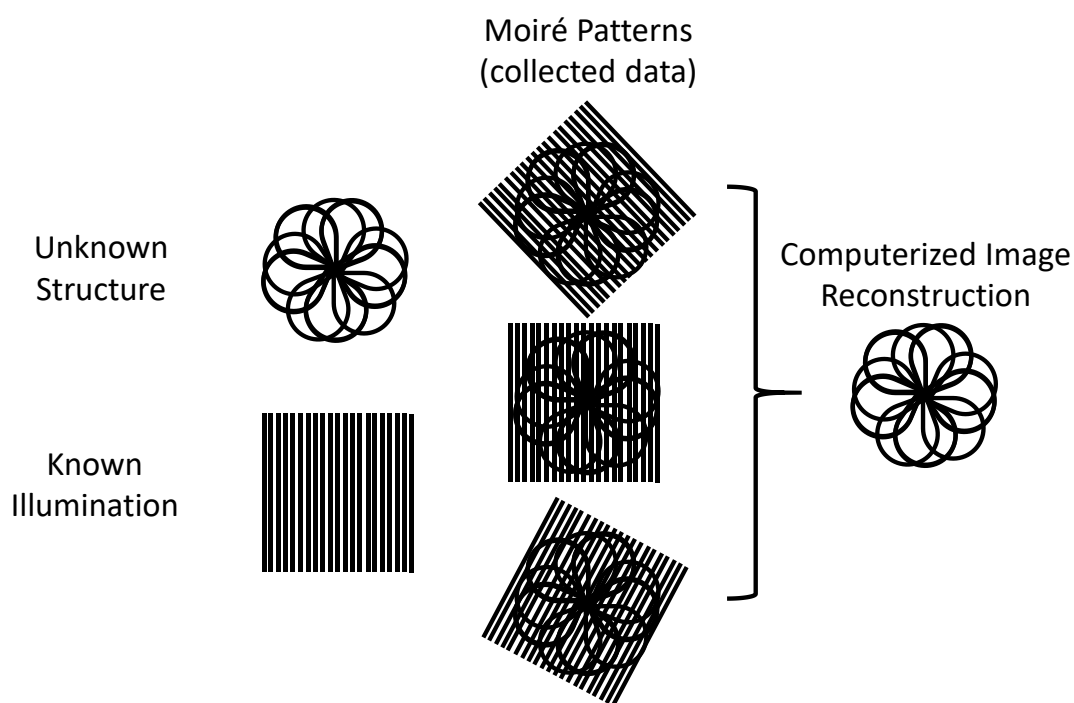


Figure 1.3. Summary of structured illumination microscopy. A specimen of unknown structure is illuminated with a known illumination pattern. This pattern is translated and rotated a number of times to generate a series of images. The resulting emission patterns (Moiré patterns) are used in conjunction with the known illumination pattern to reconstruct a final super resolution image.

While SIM obtains a poorer spatial resolution than STED or (as described below) SMLM, SIM has the advantages of a high speed and compatibility with relatively low power lasers, which are convenient for living specimens that move and may be sensitive to illumination with very intense light. Additionally, with SIM there is no inherent need for specialized fluorophores making multicolor imaging particularly convenient. However, by employing photoswitchable fluorophores¹⁶ or nonlinear structured-illumination¹⁷ with SIM the resolution can in principle be improved a theoretically unlimited amount.

1.3.3 *Single Molecule Localization Microscopy (SMLM)*

The second microscopy technique recognized with the 2014 Nobel Prize falls under the category of SMLM, or single molecule localization microscopy. Many other names and acronyms are used for the method such as STORM (stochastic optical reconstruction microscopy) and PALM (photo-activated localization microscopy)¹⁸. These techniques are routinely able to achieve ~20-50 nm resolution^{19–22}. While there are several methods to achieve super-resolution, the principles and tradeoffs of which have been compared extensively in many reviews^{23–29}, SMLM remains widely used. In SMLM, which will be the focus of the remainder of this thesis, a densely labeled, diffraction-limited region of a specimen appears as a single blurry spot using conventional imaging techniques because the blurred images of the individual fluorophores all overlap on the detector (Figure 1.4). However, the positions of the individual fluorophores can be found by imaging them sequentially—one at a time— and then finding their center positions with low uncertainty. The positions are found by switching all the fluorophores off—so they are not emitting signal to the camera—and turning only a small fraction of them on at any given time to be picked up by detectors. In practice, this switching appears like blinking stars against a black background. In reality, the blinking stars are weak signals emitted by single fluorophores and then detected by a highly sensitive camera. The blinking is usually achieved through a series of photochemical reactions between emissive and non-emissive states^{30–32}, however there are other methods for achieving blinking behavior which will be detailed in later sections. After many frames, typically thousands, a sufficient number of molecules are localized to reconstruct a high resolution image of the specimen.

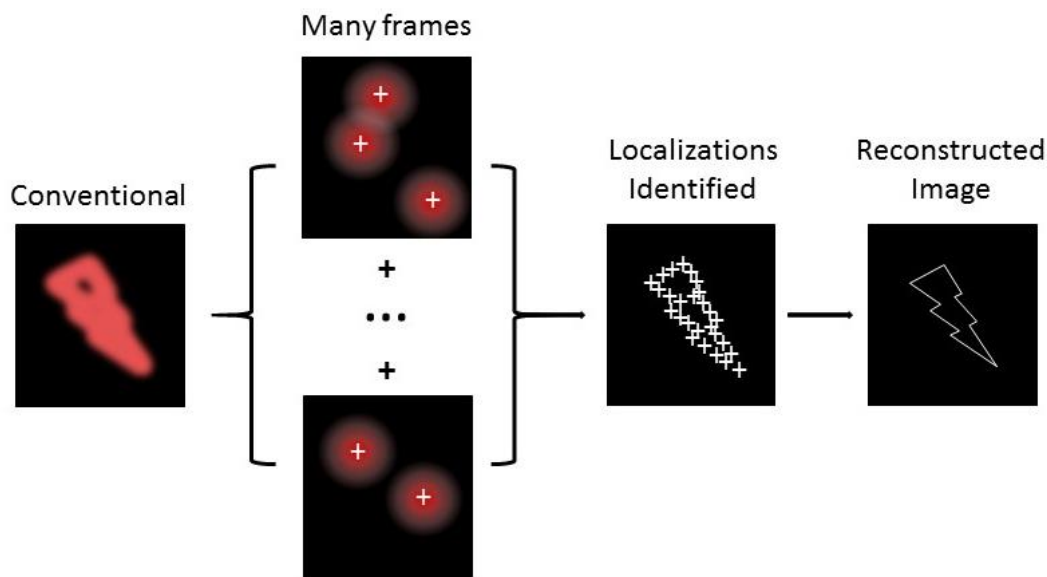


Figure 1.4. Summary of SMLM. *From left to right.* A diffraction-limited image of a sample densely labeled with fluorophores. The fluorophores are switched off and one-by-one a small subset of them are turned back on and subsequently off again or photobleached over the course of many frames. The individual fluorophores are identified and their centers are localized. After collection of many thousands of frames, the localizations are compiled into one reconstructed super resolution image.

1.4 SMLM WITH POINTS ACCUMULATION FOR IMAGING IN NANOSCALE TOPOGRAPHY (PAINT)

One of the more obvious drawbacks of traditional SMLM is the requirement for the fluorescent probe to photoswitch. A strategy to overcome this pitfall is PAINT which can achieve single molecule localizations with any fluorescent probe, not just those that photoswitch.

1.4.1 Mechanism of localization

Rather than using photochemical reactions to induce blinking of bound fluorophores, PAINT uses transient binding of fluorescently-labeled low-affinity probes to achieve blinking at the sample's focus^{33–35}. A bound probe creates a bright localizable spot, while unbound probes diffuse rapidly and create a hazy fluorescent background signal. This mechanism extends quite nicely to image multiple channels, or structures. In these cases, one structure is imaged using one low-affinity probe, then the solution is exchanged for a different low-affinity probe that binds to another structure, and this process continues until all structures of interest have been imaged³⁶. Most

commonly the low-affinity probes consist of short strands of fluorophore bearing DNA that can be tuned to have ideal affinities such that the probes bind only briefly to the target of interest before falling off and diffusing into the background but the technique is also compatible with small molecules and labeled proteins^{33,37}.

1.4.2 *Challenges with PAINT*

While its abilities to work with any fluorophore and perform well for multichannel imaging are two serious benefits of using PAINT, this method does face a serious drawback. Due to the kinetics of binding/unbinding associated with the low-affinity probes, the imaging process itself is relatively slow, especially in comparison to the imaging speeds achievable with photoswitchable SMLM. Slow imaging speeds are required because detecting single fluorophores is challenging and requires low background and the only means of increasing the binding of the probes is to increase the concentration of probes in the surrounding solution thereby increasing the background levels.

Given its slow imaging speed, PAINT has typically been limited to use with fixed specimens. This is in large part due to a live sample's ability to move during imaging. When it takes on the order of 10-20 minutes to acquire enough localizations to image a single snapshot with this approach it makes the likelihood of the sample moving during that time probable. Recently, live-cell PAINT was achieved using engineered proteins capable of binding a small emitter³⁸. This was achieved by selecting an emitter that would bind to the protein such that the rate of binding was high enough to obtain enough localizations to capture an image in less than five minutes. This, combined with careful selection of structures within the cell with low mobility, allowed them to demonstrate live-cell protein-PAINT for the first time.

1.4.3 *Future Directions of PAINT*

Although not the main focus of my research, while here at UW, I have been fortunate enough to participate on a protein design project in collaboration with the lab of Prof. David Baker (UW Biochemistry). The project received recognition for being the first of its kind to build a beta barrel protein from scratch. Prior to this work, all protein engineering of beta barrels had been

modifications of naturally occurring proteins. This is significant because beta barrel proteins are highly desirable workhorse tools for protein engineers as they provide a stable scaffold and an ability to bind small molecules. By engineering one from scratch it allows researchers more flexibility in their designs and opens the door to creating many different small molecule binding proteins in the future. In the paper I published with them, not only did they design a protein from scratch but they made it selectively bind to a small molecule which will only emit light when bound to the protein³⁹. My contribution to the project was to verify the viability of their proteins within living mammalian cells. With that, I was able to image the first ever de novo-designed beta barrel proteins in fixed and living mammalian cells (Figure 1.5, Movie 1). While the protein design work was groundbreaking, the properties of the fluorophores were unsuitable to super-resolution microscopy and my work with them was limited to conventional fluorescence microscopy. Nonetheless, the easy tunability of these designer proteins makes them highly attractive for further development, including for potential use with protein-based PAINT.

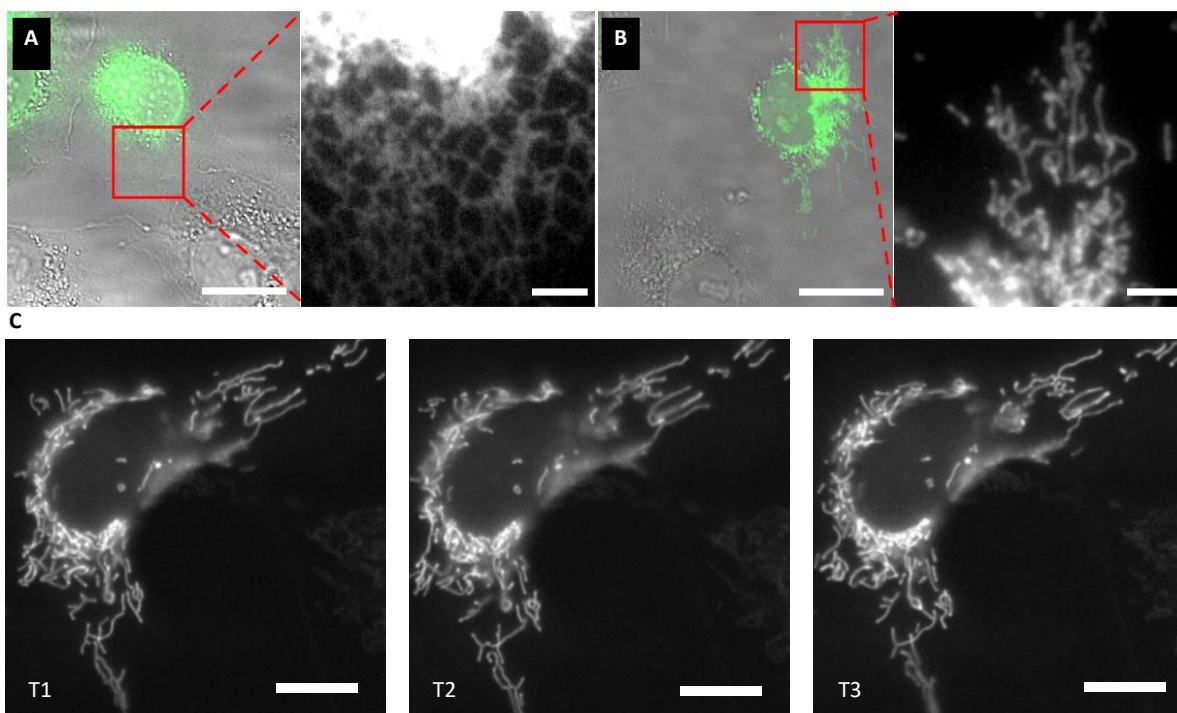


Figure 1.5. Cell imaging of de novo designed beta barrel proteins. A, B) Overlay of widefield epifluorescence (green) and brightfield (grey) images of fixed COS-7 cells expressing sec61 β -mFAP1 (A) and mito-mFAP2 (B; mito, mitochondrial targeting sequence) with expanded views of the fluorescence in the boxed regions. C) Panels taken from live-cell movie of COS-7 cells expressing mito-mFAP2 (Movie 1) at three time points (T1, T2, T3). Scale bars, 20 μ m (A, B), 3 μ m (expanded), 10 μ m (C). Figure Adapted with permission from Dou et al.

1.5 SMLM WITH PHOTOSWITCHABLE FLUOROPHORES

SMLM with photoswitchable fluorophores has been the focus of the majority of my time at UW. As such, it will be the focus of the remainder of this work.

1.5.1 Mechanism of localization

In the case of SMLM with photoswitchable fluorophores, a molecule exists in the bright emissive state and transitions to a non-emissive state, and back to a bright state prior to photobleaching (Figure 1.6)^{19,20,32,40}. This cycle occurs many times during imaging, allowing fluorophores to be

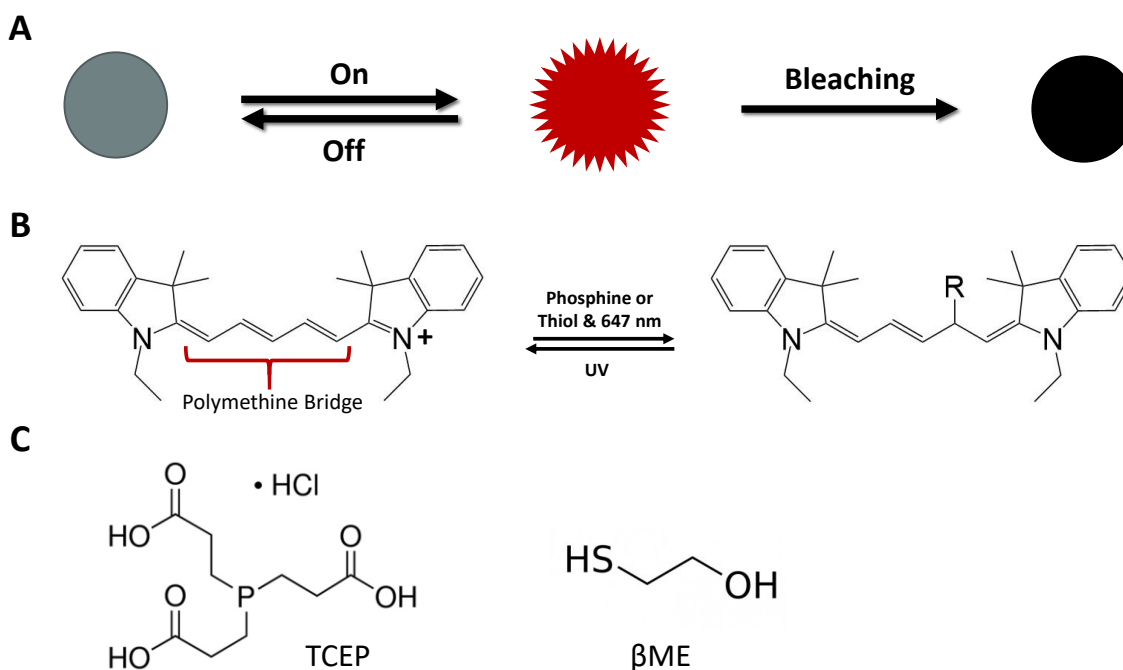


Figure 1.6. Cyanine 5 photoswitching mechanism. A) Schematic for general photoswitching mechanism of photoswitchable fluorophores. Fluorophores in a non-emissive state (grey) reversibly switch to an emissive state (red) and either convert back to the non-emissive state or convert to a permanently photobleached state (black). B) The basic structure of cyanine 5 (Cy5, where indole sulfonate groups and bioconjugation groups have been omitted). When Cy5 is illuminated with intense ~640 nm irradiation in a deoxygenated solution containing a primary thiol such as 2-mercaptoethanol (β ME) or the phosphine tris(2-carboxyethyl)phosphine (TCEP), an adduct is formed which disrupts conjugation along the polymethine bridge, causing the fluorophore to enter a non-emissive state. Upon UV irradiation, the thiol or phosphine is dissociated and fluorescence is recovered. Eventually, Cy5 photobleaches (not shown). C) Molecular structure of TCEP and β ME.

localized multiple times. One example of this is with the organic dye, cyanine 5 (Cy5), which will be used interchangeably throughout this text with the organic dye Alexa Fluor 647 due to their closeness in structures and performance in SMLM.

1.5.2 Important considerations

Unlike conventional microscopy where spatial resolution is determined primarily by an instrument's optics, in SMLM, dye switching properties determine the obtainable resolution. Two key properties determine a dye's suitability for SMLM, duty cycle and number of photons (Figure 1.7). Duty cycle is defined as the fraction of time a fluorophore spends in the on state. This is inversely proportional to the maximum density of localized fluorophores since no more than one fluorophore at a time can be localized within a diffraction-limited region. A low duty cycle is

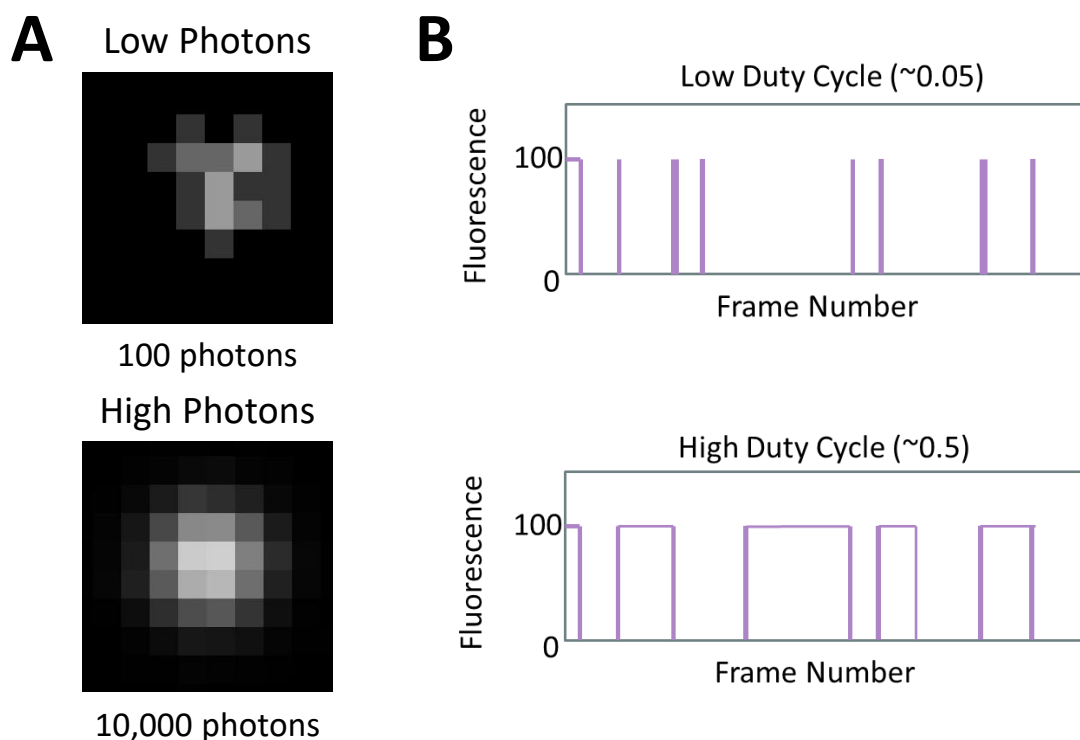


Figure 1.7. Key properties of photoswitchable fluorophores for SMLM. A) An example of a localization containing low (top) and high (bottom) numbers of photons. The high number of photons allows for a more accurate determination of the molecule's center. B) Illustration of intermittent fluorescence emission from a single molecule during the course of a movie. A molecule with a low duty cycle (top) will not be emitting signal for the majority of the movie. A molecule with a high duty cycle will be emitting signal for many more frames at a time.

desirable for SMLM because it limits the chance for more than one fluorophore to be on in a diffraction limited period during any given frame. The number of detected photons (N) limits the localization uncertainty and is approximately proportional to $1/N^{1/2}$ ^{41,42}. As N increases, the uncertainty in localization decreases. In order to reconstruct high resolution SMLM images, a low duty cycle and high photon output are required. This combination is quite rare and at present, Alexa Fluor 647 is the best known SMLM dye out of many fluorophores which have been studied to date^{18,43,44}.

1.5.3 *Challenges with photoswitchable fluorophore imaging*

Owing to the fact that Alexa Fluor 647 is the only great dye for SMLM, multicolor imaging has proven to be quite challenging with SMLM. The most obvious means of achieving multicolor imaging would be through the use of spectrally distinct dyes. Although intuitive, this approach is hindered by a lack of fluorophores in different spectral regions with excellent switching properties. Additionally, most commercial instruments lack the necessary high-power lasers to image more than two channels and the cost increase for more laser lines is a huge disadvantage for considerably worse performance at other wavelengths⁴³.

Many alternative multichannel approaches have been reported which can make use of Alexa Fluor 647 for multiple channels. These alternative approaches include the use of activator-reporter pairs⁴⁰, the use of spectrally overlapping fluorophores with ratiometric imaging⁴⁵, and the use of spectrally overlapping fluorophores with spectrally dispersed detection⁴⁶, however, all of these approaches do so by sacrificing the maximum achievable density of localizations.

Other approaches to multicolor SMLM have used sequential antibody staining and imaging techniques in which one imaging target is labeled with an Alexa Fluor 647 antibody, imaged, chemically and/or photo-bleached, and repeated in order to image multiple channels^{47,48}. These approaches are extremely tedious and/or slow (~1 day to image 3-4 channels), but they do establish the feasibility of restaining-based approaches for multichannel imaging.

While Alexa Fluor 647 is generally the preferred choice for SMLM, even its properties impose important limitations. First, most implementations of SMLM are limited to a thin focal

depth of ~600 nm, with or without the utilization of 3D localization^{49–51}, and in the process of acquiring data on one focal plane, the fluorophores in other focal planes are illuminated and photobleached. While some reports have described methods for simultaneously imaging multiple focal planes⁵² or for scanning the focal plane through the sample^{49,53}, these approaches either sacrifice photons per localization or else divide up the number of total localizations achievable among the focal planes. In Chapter 3 I will present a strategy which overcomes difficulties with 3D imaging due to photobleaching and enables rapid multichannel imaging using only Alexa Fluor 647 for all channels. The second limitation fluorophore properties impose is the requirement to image one fluorophore or fewer at a time in a diffraction-limited region. This restricts the highest achievable density and densely labeled objects either suffer a large number of false double/multiple localizations or face insufficient density of localizations which compromises the spatial resolution. I will address a solution to this limitation in chapter 4.

1.6 SUMMARY

The field of super-resolution microscopy has grown rapidly in the last decade and will continue to evolve in the next. Its popularity and recognition highlight the fact that this technology is highly desirable for those trying to investigate the littlest things in life. The famous artist, Vincent van Gogh, once wrote “Great things are not done by impulse, but by a series of small things brought together.”⁵⁴ We are at the forefront of technological advancement that is going to be able to piece together the small things that build the bigger picture. In the following chapters, this thesis discusses photoswitchable fluorophores for super-resolution imaging in greater detail (Chapter 2) and addresses the challenges of SMLM discussed above and my contribution to the field through advancement of methods for multichannel (Chapter 3), extended depth (Chapter 3) and structurally dense SMLM imaging (Chapter 4).

CHAPTER 2. TWINKLE, TWINKLE LITTLE STAR: PHOTOSWITCHABLE FLUOROPHORES FOR SUPER-RESOLUTION IMAGING

2.1 PREFACE

During my first year as a graduate student at UW, and while still helping to set up the newly established Vaughan lab, I co-first-authored a review on photoswitchable fluorophores for super-resolution imaging in the journal *FEBS Letters*. This process was a valuable as it enabled me to fully immerse myself in the field of super-resolution fluorescence microscopy. At the time the lab consisted of myself, Mr. Tyler J. Chozinski and Dr. Joshua Vaughan, and we were all highly involved with all aspects of the preparation of this review.

I spent a substantial time pouring over the literature reports and in the review in order to accurately summarize the state of the field. We then all worked on preparation of the manuscript, with my focus being primarily on the sections pertaining to properties of fluorescent probes for super-resolution imaging (**section 2.4**) and fluorescent proteins for super-resolution imaging (**section 2.5**).

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†Tyler J. Chozinski; †**Lauren A. Gagnon**; and Joshua C. Vaughan; “Twinkle, twinkle little star: Photoswitchable fluorophores for super-resolution imaging”, *FEBS Letters* **588**, 3603-3612 (2014). Copyright 2014 Federation of European Biochemical Societies.

†Authors contributed equally to the work.

All material in this chapter has been reformatted to conform to the style of this thesis.

2.2 ABSTRACT

Photoswitchable fluorescent probes are key elements of newly developed super-resolution fluorescence microscopy techniques that enable far-field interrogation of biological systems with a resolution of 50 nm or better. In contrast to most conventional fluorescence imaging techniques, the performance achievable by most super-resolution techniques is critically impacted by the photoswitching properties of the fluorophores. Here we review photoswitchable

fluorophores for super-resolution imaging with discussion of the fundamental principles involved, a focus on practical implementation with available tools, and an outlook on future directions.

2.3 INTRODUCTION

Fluorescence microscopy allows researchers to explore the inner workings of fixed or living specimens in order to directly monitor organization and dynamics occurring within a given microenvironment. Its immense popularity arises in large part from the ability to specifically label molecules or structures of interest within a cell and from its ability to study living organisms. While fluorescence microscopy has revolutionized the visualization of structures and dynamics in biology, it has traditionally faced a resolution limit of about 250 nm laterally and 500 nm axially, such that features closer than these distances are obscured. This resolution limit is caused by the diffraction of light and is a characteristic of all far-field light microscopies, where far-field here refers to configurations where a detector is placed several wavelengths or further from the emitting dipole. Other biological imaging modalities such as electron microscopy, atomic force microscopy, or near-field techniques that place a detector or imaging probe within approximately one wavelength or less of the emitting dipole, are capable of attaining very high spatial resolution but either have a poor ability to identify the molecules being examined or are primarily limited to the imaging of surface features.

Recently, however, super-resolution fluorescence imaging techniques have emerged which are able to achieve a resolution of ~50 nm or better while maintaining the high molecular specificity and live-cell compatibility of conventional fluorescence microscopy^{23,24}. These advances rely critically upon the ability to manipulate fluorophores between bright and dark states in order to reveal sub-diffraction limit spatial information about samples being studied. In this review we will focus on photoswitchable fluorophores for super-resolution fluorescence microscopy, including a discussion of how they impact imaging performance, recent developments in the field, and ongoing areas where additional work is needed. There are already many excellent reviews on super-resolution microscopy techniques and we refer the reader to these for more detailed discussion of the techniques^{23–29}.

Far-field super-resolution methods fall loosely into two categories. Techniques such as stimulated emission depletion (STED) microscopy, the related technique reversible saturable optically linear fluorescence transitions (RESOLFT) microscopy, and saturated or photoswitching structured illumination microscopy (SIM) use patterned illumination in combination with saturation effects to generate signal from only a subset of molecules within a diffraction-limited region (Figure 2.1)^{16,55–57}. In the case of STED, a donut-shaped depletion beam of wavelength longer than the detected fluorescence band is typically used to stimulate emission of light from excited state molecules at the periphery of a diffraction limited spot. When stimulated emission is driven to a saturating regime, only excited state fluorophores at the center of the donut (which has approximately zero intensity) are able to emit fluorescence. The approach is generalized in the technique RESOLFT, which can use transitions other than stimulated emission to reduce the volume from which signal is emitted. In RESOLFT this is often done by using a donut-shaped beam to photoswitch the molecules at the periphery of a diffraction-limited spot to a long-lived dark state, achieving a similar result as with STED where fluorescence is only detected from molecules at the center of the donut. The experiment for STED or RESOLFT may then be scanned and repeated over the entire region of interest to create a super-resolution image. Linear SIM¹³ is an elegant technique capable of an approximately twofold resolution improvement over conventional fluorescence microscopy, and when extended to nonlinear regimes through saturated excitation⁵⁷ or saturated photoswitching¹⁶, can in principle achieve diffraction-unlimited resolution as for STED and RESOLFT.

A second category of super-resolution imaging uses sequential localization of individual fluorescent probes to build up a high resolution image of the fluorophores labeling the sample (Figure 2.1). This is typically achieved by initially switching off the fluorescence from the fluorophores labeling the sample and subsequently activating a sparse subset of molecules to a fluorescent state. The subset of activated molecules is sufficiently sparse that one or fewer molecules is activated at any point in time within a diffraction-limited region. These activated molecules are imaged onto a sensitive camera or detector, switched off or bleached, and the process is repeated many times until all or a sufficient number of fluorophores have been recorded. The images of the activated fluorophores are individually analyzed to

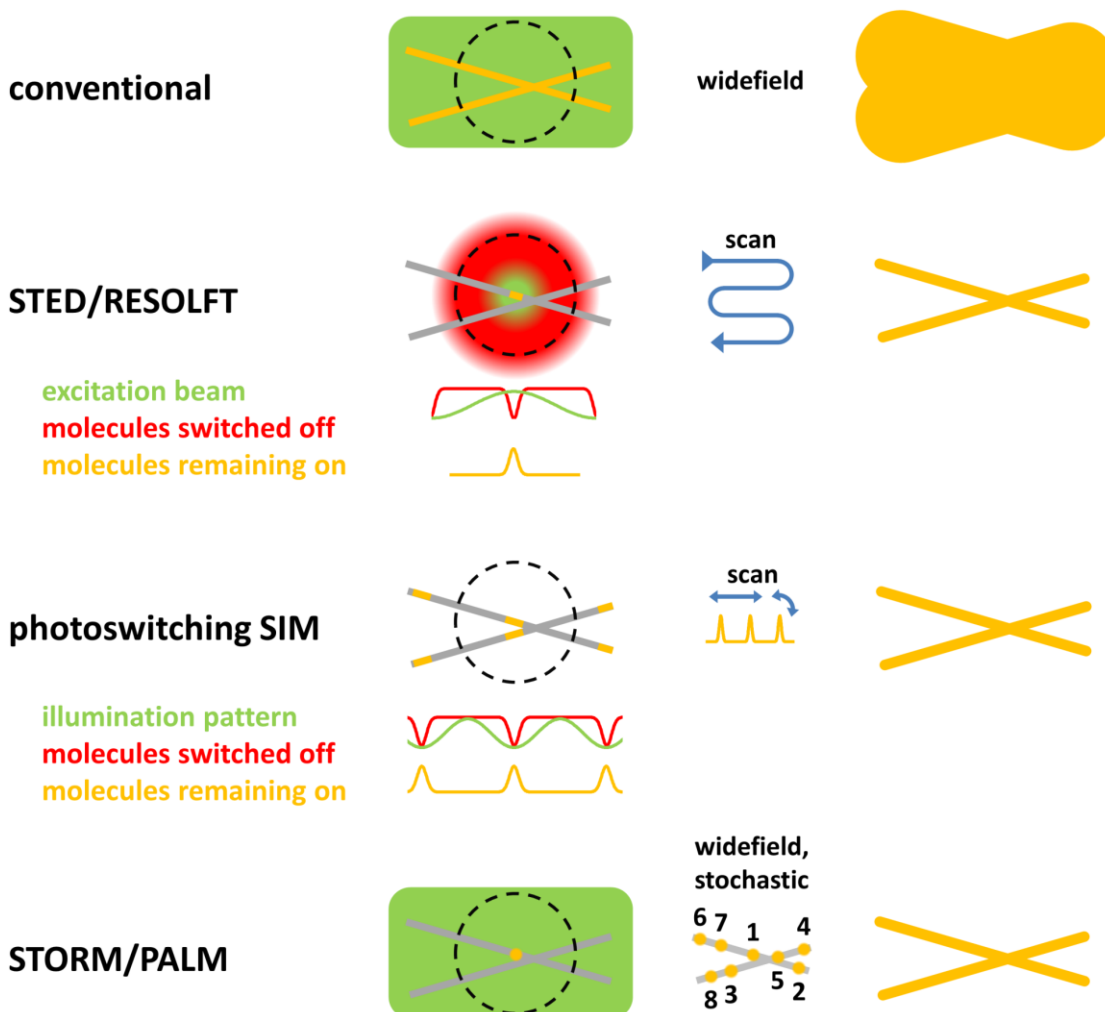


Figure 2.1. Schematics for conventional and super-resolution fluorescence microscopy. In conventional imaging, a small object consisting of two crossed lines produces a blurry image (top right) due to the diffraction of light. The dashed circle indicates the ~ 250 nm size of a diffraction limited spot which characterizes the resolution of conventional fluorescence microscopy. In STED and RESOLFT, the molecules at the periphery of the diffraction limited spot are switched off using a donut-shaped beam with saturated illumination, such that only fluorophores in the middle of the donut are visualized. Molecules are switched back on (for RESOLFT) and then the procedure is repeated and raster scanned across the sample to record a super-resolution image. In photoswitching SIM (psSIM), a saturating sinusoidal illumination pattern (green) is used to switch off all molecules except those near the minima (zeros) of the pattern. The molecules remaining on are imaged by phase shifting the sinusoidal illumination pattern by half a cycle and then the molecules are switched back on with illumination at a new wavelength. The sinusoidal pattern is then scanned over the sample at different phases and angles, and the full image series is analyzed to extract a super-resolution image. In STORM/PALM, individual fluorophores (numbered 1-8) are sequentially activated, imaged, and bleached using widefield illumination, and a super-resolution image is reconstructed from fitted positions of the individual fluorophores.

determine their positions with low uncertainty^{41,58}, and a final image is reconstructed from the determined positions of the fluorophores. The technique is known as stochastic optical reconstruction microscopy (STORM)¹⁹, photoactivated localization microscopy (PALM)²², fluorescence PALM (FPALM)²⁰, and numerous subsequent acronyms, but all share the same basic single-fluorophore approach. For brevity, we will refer to this family of techniques simply as STORM/PALM.

Despite substantial differences in implementation, these far-field super-resolution techniques share several common features. Fundamentally, they all use some sort of switching mechanism to reduce the volume of the emitting signal, where the single molecule approach may perhaps be seen as limit of the smallest possible emitting volume. The reduction of the emitting volume is achieved in most super-resolution techniques by photoswitching fluorophores between long-lived bright and dark states, and this is used for STORM, psSIM, and most implementations of RESOLFT. As such, there are special requirements on the fluorophores used in these methods which have not previously been important for conventional fluorescence microscopy techniques. It is important to note that STED does not switch off fluorescence by means of long-lived dark states, but does use light to suppress signal for most excitation events, and

that this also places

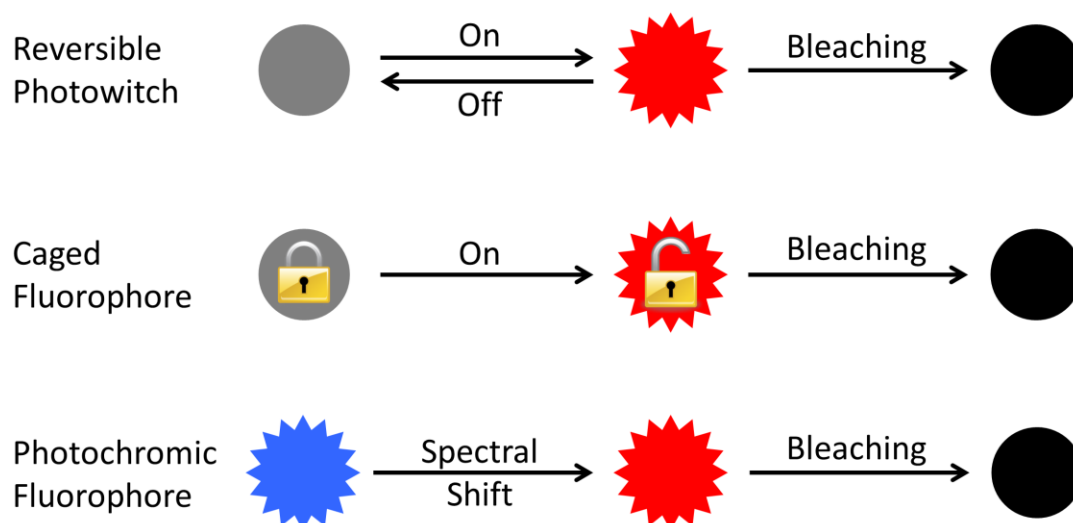


Figure 2.2. Overview of different types of photoswitching used in super-resolution fluorescence microscopy. Gray circles indicate non-emissive fluorophores, while black circles indicate bleached fluorophores. Red and blue stars indicate fluorophores emitting red or blue light.

specific demands on fluorophores. Figure 2.2 provides an overview of the different types of photoswitching used in super-resolution fluorescence microscopy.

2.4 PROPERTIES OF FLUORESCENT PROBES FOR SUPER-RESOLUTION IMAGING

Photoswitchable fluorophores are relative newcomers to bioimaging; however, they have made appearances in the literature well before the advent of super-resolution imaging. For example, Mitchison created a photoactivatable fluorescein derivative covalently linked to tubulin and subsequently used this to observe microtubule polymerization at kinetochores^{59,60}. Although photoswitches are becoming more popular among the biological imaging community, photoswitches have been known for decades for non-bioimaging applications (e.g., diarylethenes, spiropyran, etc.)^{61,62}. Several key parameters describe the photoswitching behavior, including on-off contrast ratio, number of switching cycles, duty cycle, photons per switching event, switching cycle rate, dependence of these properties on illumination intensity and chemical environment. These parameters are in addition to general fluorophore properties to consider, including extinction coefficient, quantum yield, absorption and fluorescence wavelengths, photostability, and water solubility, etc.

The *on-off contrast* is the brightness of the fluorophore in the ‘on’ state divided by that of the ‘off’ state. This property impacts the ability to generate contrast between the fluorophores in the reduced volume of a diffraction-limited region compared to that of the remainder of the fluorophores in the diffraction limited region. If the on-off contrast ratio is low (poor), then it is difficult to detect fluorescence from a small sub-region of a diffraction-limited spot since the fluorophores outside of that sub-region would provide a large competing signal that effectively blurs out the measurement. For this reason, a high on-off ratio is generally desired. The analogue in STED is determined by efficiency of the donut-shaped depletion beam in suppressing fluorescence from the excited state fluorophores.

Photoswitchable fluorophores typically transit between bright and dark states some number of times before irreversibly photobleaching. The *number of switching cycles* achievable with a given fluorophore and/or condition imposes limitations depending on the imaging modality. For techniques like RESOLFT or psSIM, the fluorophores in a given diffraction limited

region must be photoswitched many times in order to record a single super-resolution image. The analysis routines used in these techniques also typically assume that there is little photobleaching during the acquisition of a set of data to generate one super-resolution image, so it is desirable to have a photoswitchable molecule which can be switched many times. For STORM/PALM, a single cycle of photoswitching is sufficient, though the use of several switching cycles does improve image resolution by averaging out stochastic localization errors⁴³. Fluorophores in STED must also be able to be scanned many times, and premature bleaching may be problematic due to the possibility of the intense STED beam exciting fluorophores to highly excited states that are rapidly bleached. Additionally, when imaging dynamics in living specimens or specimens which change over time, many switching cycles are generally desired for all of the super-resolution imaging techniques. On the other hand, for STORM/PALM, a single photoactivation followed by irreversible photobleaching would in principle provide a particularly clean way to report on numbers of dye molecules decorating a sample and, with sufficiently well-characterized labeling strategies, could directly report on analyte stoichiometry. Despite the present lack of a 'perfect' single-cycle photoswitch, however, strategies which account for multiple photoswitching cycles have nonetheless made good progress in determining stoichiometry^{63–65}.

Two properties of photoswitches are especially important for STORM/PALM, namely photons per switching event and duty cycle. The *number of detected photons* per switching event is typically the main factor dictating the uncertainty of determining a fluorophore's position, where the uncertainty scales roughly as the inverse square root of the number of detected photons^{41,42}. In a sense, this uncertainty determines the reduced volume achieved for STORM/PALM compared to the diffraction limited region (Figure 2.1), but does not solely determine the achievable resolution, since it does not factor in the achievable density of labels. It is the *duty cycle*, or fraction of time a fluorophore spends in an on state, which limits the maximum density of fluorophores that may be localized in a diffraction-limited region, with approximately an inverse relationship between fluorophore density and duty cycle⁴³. This limit on fluorophore density is a simple consequence of the Nyquist sampling theorem, which states that the maximum attainable resolution is equal to twice the average distance between adjacent

probes⁶⁶. Together, photons per localization and duty cycle limit the spatial resolution achievable by STORM/PALM.

The on and off *switching rates* limit the speed with which super-resolution images may be recorded and are important factors governing the temporal resolution achievable with super-resolution imaging. Slower photoswitching rates require more acquisition time while faster photoswitching rates accommodate faster data acquisition. In many cases the photoswitching rates are controllable by illumination at one or more wavelengths or by changing the chemical composition of the imaging cocktail (see below).

Additional properties are worth noting, although we will not discuss them in detail here. Illumination of biological specimens with intense visible or ultraviolet light can lead to phototoxicity, in particular when labeled with fluorophores that can produce reactive oxygen species, and it is important for live-cell imaging experiments to assess potential artifacts due to phototoxicity⁶⁷. For STORM/PALM, errors in position determination may occur if fluorophores are not freely rotating on the time scale of the measurements, especially for molecules observed slightly above or below the optimum focus^{30,68–70}. Compatibility with multicolor imaging is another key property. For example, some popular STORM/PALM fluorophores, such as EosFP and its relatives, initially fluoresce in the green region of the spectrum (~515 nm) and photoconvert to fluoresce in the orange/red (~575 nm, see also Figure 2.2). This means that EosFP consumes two emission channels, leaving less options available for multicolor imaging, and thus motivating the search for fluorophores which maintain all the other desirable qualities but which do not cause spectral interference^{66,71}.

With all of these important fluorophore properties for super-resolution imaging, it should not be a surprise that there is a large amount of variability in fluorophore performance. Stefan Hell's group has assembled a list of many fluorophores used in STED along with the reported resolution and the conditions used (e.g. depletion wavelength and pulse length)⁷². The attainable resolution varies between 16-140 nm, and perhaps future efforts will help to provide insights into what properties of the fluorophores lead to better or worse resolution. Recent work has systematically evaluated many organic fluorophores and fluorescent proteins for use in STORM/PALM, and as with STED, some STORM/PALM fluorophores achieved excellent results

while others performed poorly or were practically useless^{43,73}. This is in stark contrast to conventional fluorescence microscopy, where most fluorophores with good general properties (i.e., high extinction coefficient, quantum yield, and photostability) work very well.

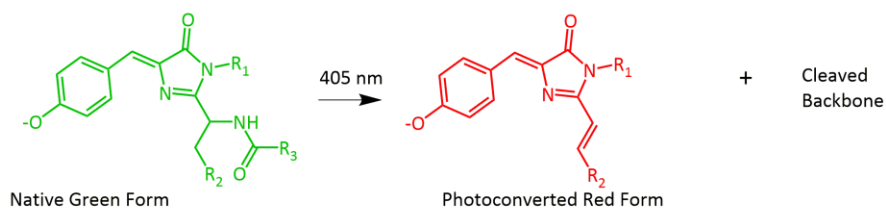
While fluorophore structure strongly impacts photoswitching performance, in many cases, the chemical environment of the fluorophores during imaging (which we refer to as the ‘imaging cocktail’) also exerts a strong influence on performance. For instance, in the case of the fluorophore Cy5 (which is a close structural relative of AlexaFluor 647, and which we use synonymously with AlexaFluor 647 in this paper) there are at least five substantially different imaging cocktails which have been reported in the literature. The original STORM/PALM publication with Cy5 used an imaging cocktail containing an oxygen scavenger system and thiol to facilitate photoswitching¹⁹. Subsequently, it has been shown that Cy5 may be: switched on and off using redox chemistry with ascorbic acid⁷⁴; reduced with NaBH₄ to a long-lived nonfluorescent form and photoactivated³⁰; switched on and off using the phosphine TCEP (tris 2-carboxyethyl phosphine)³¹; switched on and off using a thiol and a mixture of either cyclooctatetraene or unknown additives in the proprietary sample mounting medium Vectashield^{75,76}. For these different schemes, photons detected per localization varied between ~100-1,000,000, and duty cycle, which is generally harder to measure, in the neighborhood of 10^{-2} - 10^{-6} . On the one hand, the ability to tune the photoswitchable properties of a given fluorophore is incredibly powerful, and yet on the other hand presents a liability if the imaging cocktail is incorrectly formulated.

2.5 FLUORESCENT PROTEINS FOR SUPER-RESOLUTION IMAGING

Moerner and coworkers reported GFP and YFP as the first single molecule photoswitches over a decade before super-resolution microscopy techniques became widespread⁷⁷. In the years since the discovery, significant advances have been taken in discovering or developing new fluorescent protein photoswitches and mutants with characteristics tailored for specific experiments, including super-resolution fluorescence imaging applications. Several recent reviews summarize many of these developments in the context of super-resolution imaging⁷⁸⁻⁸².

In general, fluorescent proteins have the great advantages of a relatively small size (compared, for instance, to fluorescently labeled antibodies), convenient genetic encodability for tagging proteins or molecules which are bound by proteins, and excellent compatibility with imaging living samples. The photostability or number of photons per switching cycle of fluorescent proteins tends to be lower than that of popular small organic dyes and multicolor super-resolution fluorescence imaging with fluorescent proteins is still challenging. In contrast, the duty cycle of many fluorescent proteins may be 1-3 orders of magnitude better than that of popular organic fluorophores^{43,73}. Furthermore, as with any experiments involving tagged proteins, practitioners should be cautious to avoid problems related to overexpression, codon optimization, oligomerization or aggregation, perturbation of sensitive proteins, or endogenous untagged protein that may be unobservable in an experiment⁷³. Depending on the experiment, the maturation time and the impact of the tag on expression levels of the fusion protein may also be important considerations⁷³.

Photoconversion



Photoswitching

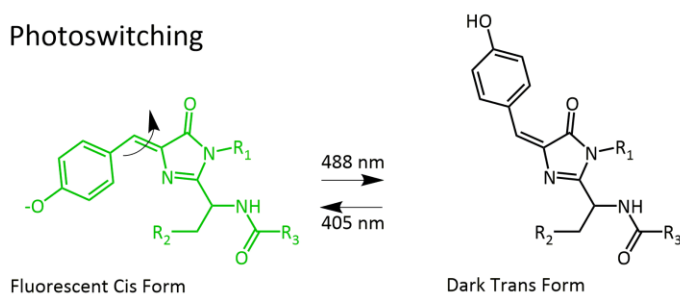


Figure 2.3. Representative photoswitching mechanisms of fluorescent proteins. A) FPs such as EosFP convert irreversibly from a green fluorescent form to a red fluorescent form upon illumination with violet or ultraviolet light. The photochromic shift occurs due to cleavage of the peptide backbone which extends the conjugation of the chromophore and red-shifts the absorption and emission spectra by 50-60 nm. B) FPs such as Dronpa undergo reversible *cis-trans* isomerization, where the *cis* form is fluorescent and the *trans* form is dark. Other fluorescent proteins may use a combination of these phenomena, such as IrisFP⁸³.

Fluorescent proteins used in super-resolution fluorescence microscopy come in several broad categories. Those used in STED must resist photobleaching while illuminated with the relatively intense excitation and depletion beams. In contrast, the fluorescent proteins used in RESOLFT, psSIM, and STORM/PALM must be photoswitchable in some way. This includes fluorescent proteins which are initially dark and may be photoactivated either irreversibly (such as photoactivatable GFP) or reversibly (such as Dronpa)^{84,85}. Other fluorescent proteins, such as EosFP, are instead able to irreversibly convert from a green fluorescent form to a red-fluorescent form, such that when viewed in the red channel, the fluorescent protein appears to photoactivate⁸⁶. While the green to red photoconversion is irreversible, many of these fluorophores also exhibit blinking behavior in the red form which may confuse quantification efforts if not properly handled^{87,88}. There are also fluorescent proteins which exhibit a combination of these two behaviors, such as IrisFP which is photoconvertible between green and red fluorescent states and also exhibits reversible photoactivation in both the green and red fluorescent forms⁸³. The basic switching behaviors are illustrated in Figure 2.3 and a table summarizing photoswitching properties of a number of fluorescent proteins are shown in Table 2.1.

STED microscopy has utilized many fluorescent proteins in super-resolution imaging to date. GFP, YFP, citrine and others have been used for ~50-70 nm resolution imaging with good results in both fixed and living cells⁸⁹⁻⁹⁷. In a particularly impressive demonstration, YFP was used to image dendrites in the brain of a living mouse at <70 nm resolution⁹⁸. Multicolor imaging with STED faces the challenge of accommodating different excitation and depletion wavelengths along with the excitation and emission bands of each fluorophore. One approach to this challenge has been to use common excitation and depletion wavelengths for two fluorophores with nearby emission bands that are separable using spectral unmixing techniques, for instance using the GFP/YFP pair where ~100 nm resolution with ~10% crosstalk was achieved⁹⁹.

Numerous reversible photoswitchable fluorophores have been utilized for RESOLFT and photoswitching SIM, including several recent excellent additions to the arsenal. Compared to imaging by STED, these probes can operate at orders of magnitude lower illumination intensities, such that phototoxicity concerns for imaging living samples are greatly reduced. Dronpa has been

Fluorescent Proteins Used in Super-resolution Imaging											
STED	notes	λ_{ex}	λ_{em}	λ_{STED}	ϵ (M^{-1}	Φ	resolution		References		
fluorophores		(nm)	(nm)	(nm)	cm^{-1}		reported				
EGFP	m	490	510	575	53,000	0.6	~70 nm		89–92		
EYFP	m	514	527	598	36,500	0.63	<100 nm		89,92,93		
Citrine	m	516	529	592	77,000	0.76	<60 nm		89,94,95		
E2Crimson	t	611	646	760	126,000	0.23	<100 nm		89,96		
TagRFP657	m	611	657	750	34,000	0.1	~74 nm		89,97		
RESOLFT/ psSIM	notes	λ_{ex}	λ_{em}	$\lambda_{\text{on/off}}$	ϵ (M^{-1}	Φ	on-off	N	References		
fluorophores		(nm)	(nm)	(nm)	cm^{-1}		contrast				
Dronpa	m, ps	503	518	405/48	95,000	0.85	~30	60-70	16,108,109		
rsEGFP	m, ps	493	510	405/49	47,000	0.36	50	>1,100	100,102		
rsEGFP2	m, ps	478	503	430/48	61,300	0.3	ND	>2,000	102,103		
Dreiklang	m, ps	511	529	340/41	83,000	0.41	75	>160	80,101		
rsCherryRev1.4	d, ps	572	609	430/59	ND	ND	ND	>1000	103		
STORM/ PALM	notes	λ_{ex}	λ_{em}	$\lambda_{\text{on/off}}$	ϵ (M^{-1}	Φ	on-off	N	phot.	duty	References
fluorophores		(nm)	(nm)	(nm)	cm^{-1}		contrast			cycle	
PAmcherry1	m, pa	564	595	~370 on 405/48	18,000	0.46	4,000	1 (1)	~725	8×10^{-6}	71,73,88
Dronpa	m, ps	503	518	8 340/41	95,000	0.85	ND	60-70	120	8×10^{-4}	16,73,78,108
Dreiklang	m, ps	511	529	2 405/57	83,000	0.41	75	ND	700	ND	80,101,110
MEos2	wd, pc	569	581	3 405/57	41,000	0.55	ND	1 (2.8)	0	3×10^{-6}	73,88,104–106
MEos3.2	m, pc	572	580	2 380/56	32,200	0.55	ND	1 (2.4)	0	3×10^{-6}	73,88,107
mMaple	wd, pc	566	583	6 405/56	30,000	0.56	ND	1 (3.4)	0	2×10^{-6}	73,88,106
mMaple2	m, pc	566	583	6	ND	ND	ND	ND	~800	1×10^{-6}	73

Table 2.1. A selection of fluorescent proteins used for super-resolution fluorescence imaging. In the notes field, each fluorescent protein's oligomeric state is indicated (m=monomer, d=dimer, wd=weak dimer, t=tetramer) along with a code for the type of fluorophore (ps=reversibly photoswitchable, pa=photoactivatable, pc=photoconvertible). The properties of the fluorescent forms of these fluorophores used for super-resolution imaging are tabulated here: λ_{ex} (excitation wavelength); λ_{em} (emission wavelength); $\lambda_{\text{on/off}}$ (wavelengths for switching the fluorescence on and off, respectively); ϵ (extinction coefficient); Φ (fluorescence quantum yield); on-off contrast ratio (ratio of intensity of the emissive form to the dark form); N (number of switching cycles, including reported blinking events after irreversible photoconversion⁸⁸); phot. (number of detected photons per localization event); ND indicates the parameter was not determined.

used for photoswitching SIM, and, under optimized conditions that yielded ~65 switching cycles, have achieved <50 nm resolution of various in vitro or cellular structures¹⁶. In 2011, Stefan Hell, Stefan Jakobs, and coworkers reported two newly developed photoswitchable fluorescent proteins, dubbed Dreiklang and reversibly switchable enhanced GFP (rsEGFP), which can photoswitch more than 160 and 1000 cycles, respectively^{100,101}. Using these low-fatigue probes, they achieved <40 nm resolution imaging in fixed cells. Subsequently, these proteins and some more recently developed ones such as rsEGFP2, rsCherryRev1.4, and others have found application in one- and two-color RESOLFT imaging in fixed or living cells at 50-100 nm resolution^{102,103}. Many of the reversibly photoswitchable fluorescent proteins are switched off using the same wavelength as the imaging wavelength and are switched on with ultraviolet light.

For STORM/PALM, EosFP and many of its close relatives have been popular choices for fluorescent labels owing to their good on-off contrast, duty cycle, and relatively high value number of detected photons per localization (~1000 photons)^{104–107}. The original tdEosFP was an obligate tetramer and has been extensively engineered to create variants which have little to no tendency to aggregate but which still maintain the desirable optical properties of the chromophore^{86,104,107}. The reversibly photoswitchable fluorescent protein Dreiklang, also described above for RESOLFT (or psSIM, in principle) emits ~400-700 detected photons/localization for many cycles and ~35 nm localization uncertainty, is particularly interesting for live-cell applications where many snapshots would be desired¹⁰¹. Other variants include mMaple2 which, like the EosFP variants, emits a good number of photons (~800 detected photons/localization) but shows a superior efficiency of expression/folding/maturation and lacks the tendency to form dimers of the original mMaple variant^{73,106}. A major current limitation is that the options for two-color (or more) STORM/PALM using fluorescent proteins are at present quite limited. This limitation arises in part since photoconvertible fluorescent proteins are preferred due to their relatively high photons/localization but also has the consequence that the two main fluorescent protein channels are consumed by a single probe.

2.6 ORGANIC FLUOROPHORES FOR SUPER-RESOLUTION IMAGING

Intermittent emission from single organic fluorophores was observed in many of the earliest single molecule fluorescence measurements^{108,109}. Approximately a decade later, the particularly robust Cy5 photoswitch was reported and then applied to super-resolution imaging by STORM/PALM^{19,110,111}. Over the next few years, several groups reported the use of a range of established organic fluorophores for imaging by STORM/PALM, with a fair amount of variability in procedures and performance^{112–114}. Meanwhile, STED, which utilizes the universal property of stimulated emission rather than photoswitching to long-lived dark states, had been demonstrated with many organic dyes⁸⁹. RESOLFT/psSIM have been less utilized with organic fluorophores, and we will omit these from our discussion on organic fluorophores except to point out here that low switching intensity organic fluorophores with a large number of switching cycles would be particularly helpful for these techniques.

In general, the best organic fluorophores substantially brighter and resistant to bleaching than their best fluorescent protein counterparts and also have the advantage of small size. For instance, the dye Abberior STAR 635P can achieve a resolution in STED which is approximately two times better than the best resolution achievable with any of the fluorescent proteins, presumably due to a high photostability^{115,116}, and in STORM/PALM, the dye Cy5 gives several times more photons per localization than any of the currently preferred photoswitchable fluorescent proteins^{43,105–107}. Because there are a wide array of organic fluorophores spanning the visible and near infrared region of the electromagnetic spectrum, there are many good options for multicolor imaging of cellular structures for both STED and STORM/PALM^{43,112,115,117,118}. Notably, the duty cycle of many photoswitchable fluorescent proteins is 10-1000 times lower than many of the currently favored photoswitchable organic fluorophores, and may enable experiments at higher densities with photoswitchable fluorescent proteins than is achievable with photoswitchable organic fluorophores^{43,73}.

Organic fluorophores are not without their drawbacks, however, and in many cases have inferior properties for labeling samples compared to fluorescent proteins. The use of indirect or direct immunofluorescence with bulky dye-conjugated antibodies may blur out structures of interest and in most cases requires fixation and permeabilization of the specimen to

accommodate staining. The advent of small, single-chain antibodies raises the prospect of using small epitope tags with minimal blurring effects¹¹⁹ although these are at present only commercially available for targeting a very small number of proteins or epitope tags and in any event are primarily restricted to use in nonliving specimens. Some organic fluorophores are able to label specific cellular structures of interest in live or fixed specimens by virtue of the intrinsic properties of the fluorophore or an attached moiety^{120,121}, but the approach is limited to fairly a small subset of organelles or structures (e.g. mitochondria, the plasma membrane, cytoskeleton, etc.). A growing body of labeling techniques utilizes reactive protein tags that can recognize a fluorophore conjugated to a substrate which may be bound by or covalently linked to the reactive protein^{122–124}. While these reactive labeling techniques offer the prospects of the best of both worlds, at present, many of the desirable organic fluorophores are not cell-permeant and thus practitioners may be restricted to the use of cell-permeant fluorophores or the use of dye delivery of impermeant fluorophores by bead loading, pinocytosis, or electroporation¹⁰⁵.

Organic fluorophores photoswitch by a variety of mechanisms. For instance, Cy5 has been shown to react with thiols upon illumination with red light, or with the phosphine TCEP (tris 2-carboxyethylphosphine) in the absence of light, to generate a nonfluorescent adduct^{31,32}. The adduct consists of the addition of the thiol or phosphine to the polymethine bridge of Cy5 such that the conjugation of dye is disrupted and the molecule's absorption shifts to the ultraviolet (Figure 2.4). In contrast, several rhodamine fluorophores have been shown to generate long-lived nonfluorescent radical species which can also be photoactivated by illumination with ultraviolet light¹²⁵. Yet a third mechanism for photoswitching has been established for the oxazine dye Atto 655, which undergoes two one-electron reactions to generate a reduced form of the dye and which is also activatable by illumination with ultraviolet light^{125,126}. Some fluorophores are prepared chemically in a dark form and may be photoactivated by illumination with, for instance, ultraviolet light^{30,127–131}. The details of many of these chemical or photochemical reactions require further study. These photoswitching properties have been primarily utilized for STORM/PALM, to date. As mentioned earlier, in STED, it is stimulated emission of molecules at the periphery of a diffraction limited region which leads to reduction of the imaging volume. While stimulated emission is a process common to all fluorophores, transitions to higher lying

states that can lead to premature photobleaching tends to be one of the key factors that renders a fluorophore unsuitable for STED.

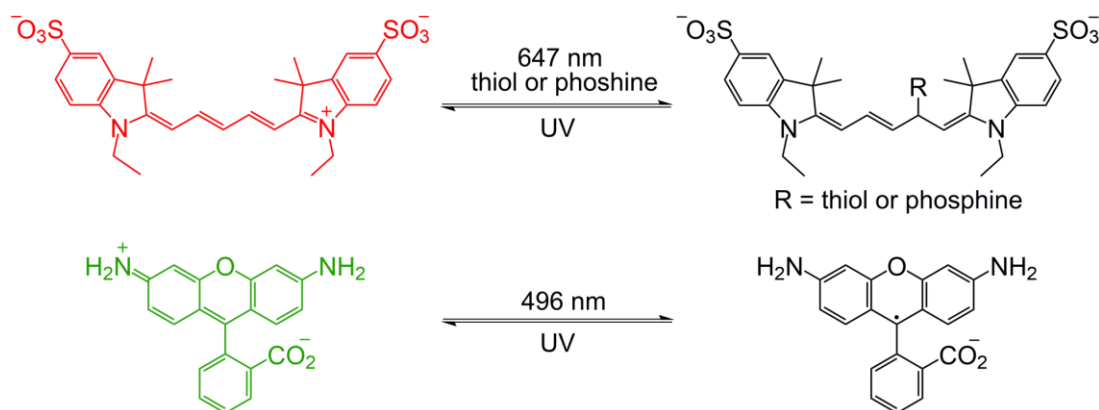


Figure 2.4. Example mechanisms for photoswitching of a cyanine and rhodamine fluorophore.

(Top) Upon incubation with a phosphine or illumination with 647 nm light in the presence of a thiol or phosphine, Cy5 transitions to a nonfluorescent state adduct consisting of the phosphine or thiol bound to the polymethine bridge. Illumination with ultraviolet light can dissociate the adduct. (Bottom) Several rhodamine fluorophores can be converted to a long-lived, nonfluorescent radical upon illumination with light at their main absorption band in the absence of oxygen. The dark form may be photoactivated by illumination with ultraviolet light.

STED has been used with a wide range of organic fluorophores and many recent developments have in particular improved the technique's multicolor capabilities. These include long-established STED fluorophores such as Chromeo 488, Atto 565, and Atto 647N, as well the recent rhodamine derivatives, carbopyronine derivatives, or Abberior STAR series fluorophores which are now commercially available (e.g., Abberior STAR 635P)^{72,89,115,116,132,133}. Multicolor imaging strategies include the use of common excitation and depletion wavelengths with closely emitting fluorophores (e.g., that can be separated through spectral unmixing techniques, as was described above for two-color fluorescent protein imaging). A second strategy utilizes two fluorophores with well-separated excitation and emission spectra (e.g., Atto 590 and Abberior STAR 635P) whose fluorescence emission can be switched off with a common depletion beam¹³⁴. The use of a common depletion beam in both of these approaches helps to ensure good channel registration and also helps minimize complexity and cost in the apparatus.

A wide range of organic fluorophores have also been employed in STORM/PALM, including representatives from many popular general classes of fluorophores such as rhodamines, cyanines, oxazines, carbopyronines, bodipy fluorophores and others^{43,112–114,120,131,135}. The results obtainable with the fluorophores vary widely, however, and as with STED fluorophores, it is recommended to choose STORM/PALM fluorophores carefully. A recent systematic comparison of many commercially available organic fluorophores for STORM/PALM⁴³ established good metrics for evaluating fluorophore fitness and identified several very good fluorophores amongst the panel and conditions tested. One key finding of interest to practitioners is that when using the popular oxygen scavenger/thiol based imaging cocktail, the best results amongst the explored dyes and conditions were obtained with Cy5 (or AlexaFluor 647) and the best four-color results were obtained with Atto 488/Cy3B/AlexaFluor 647/DyLight 750. Subsequent reports from the same group include an improved two-color imaging scheme based on phosphine quenching of cyanine fluorophores³¹, organelle/membrane probes for live-cell STORM/PALM imaging¹²⁰, and reductively caged fluorophores which when activated in an optimized imaging cocktail^{136,137} can yield 10-1000 times greater photons per localization³⁰. Data for a subset of these fluorophores are summarized in Error! Reference source not found..

Beyond utilizing long-established, commercially available fluorophores, there has been growing interest in the development of new fluorophores or fluorophore variants that are tuned for one or more super-resolution methods, including STORM/PALM. Amongst these fluorophores are various caged rhodamine or fluorescein derivatives^{129–131,138} and fluorophores based on DCDHF (2-dicyanomethylene-3-cyano-2,5-dihydrofuran)¹³⁹. As super-resolution imaging techniques become more widespread and demand grows for excellent super-resolution probes, hopefully these and other optimized compounds become commercially available in order to have broad impact in the hands of researchers.

In contrast to fluorescent proteins, which are typically insensitive to the composition of the imaging solution, organic fluorophores are very sensitive, both in terms of general fluorescence properties (e.g., photostability) and in terms of photoswitching properties. The use of various additives to form an ‘imaging cocktail’ is an important part of many of these techniques, but often the effects are difficult to interpret since the additives may function in

Organic Fluorophores Used in Super-resolution Imaging										
STED fluorophore	notes	λ_{ex} (nm)	λ_{em} (nm)	λ_{STED} (nm)	ϵ (M ⁻¹ cm ⁻¹)	Φ	resolution reported	References		
Chromo 488		488	517	592	73,000	0.38	30 nm	89,120,147		
Chromo 494		494	628	760	55,000	0.28	~80 nm	89,147,148		
Atto 565		563	592	~662	120,000	0.9	20-50 nm	89,120,149–151		
Atto 647N		644	669	~765	150,000	0.5	30-60 nm	89,120,149–151		
Fl-Rhodamine		633	653	~762	75,000	0.92	~40 nm	118,119,136,152		
Si-Rhodamine		645	662	~775	100,000	0.39	~50 nm	153		
AbberiorSTAR635P		635	655	~775	75,000	0.92	~20 nm	118,119		
STORM/PALM fluorophore	notes	λ_{ex} (nm)	λ_{em} (nm)	$\lambda_{\text{on/off}}$ (nm)	ϵ (M ⁻¹ cm ⁻¹)	Φ	duty cycle	N	phot.	References
Atto 488 (thiol)	ps	501	523	405/488	90,000	0.8	0.0022	49	1,100	43,151
Atto 488	pa	501	523	405/488	90,000	0.8	0.002	~1	~10 ⁴	30,43,151
Cy3B (thiol)	ps	559	570	405/561	130,000	0.67	0.0004	5	2050	43,154
Cy3B	pa	559	570	405/561	130,000	0.67	0.0004	~1	~10 ⁵	30,43,154
Alexa 647 (thiol)	ps	650	665	405/647	239,000	0.33	0.0007	26	5,200	43,155
Cy7 (thiol)	ps	747	776	405/750	250,000	0.28	0.0004	~3	1,000	43,154
Alexa 750 (TCEP)	ps	749	775	405/750	240,000	0.12	0.0004	ND	2,800	31,43,155
Si-Rhod. (thiol)	ps	645	662	ND/640	100,000	0.39	ND	ND	630	153

Table 2.2. A selection of small organic fluorophores used for super-resolution fluorescence imaging. In the notes field for STORM/PALM fluorophores, a two letter code for the type of fluorophore is indicated in each case (ps=reversibly photoswitchable, pa=photoactivatable). For photoswitched fluorophores in the STORM/PALM section, the use of a thiol or phosphine switching agent is indicated by (thiol) or (TCEP, for tris 2-carboxyethylphosphine), while photoactivated/caged fluorophores lack the designation. The properties of the fluorescent forms of these fluorophores used for super-resolution imaging are tabulated here: λ_{ex} (excitation wavelength); λ_{em} (emission wavelength); $\lambda_{\text{on/off}}$ (wavelengths for switching the fluorescence on and off, respectively); ϵ (extinction coefficient); Φ (fluorescence quantum yield); duty cycle (fraction of time spent in the on state); N (number of switching cycles, including reported blinking events after irreversible photoconversion⁸⁸); phot. (number of detected photons per localization event); ND indicates the parameter was not determined. In most cases, on-off contrast values were not reported. Comparably few reports have been published for RESOLFT/psSIM and this section has not been tabulated here.

multiple ways. For example, thiols are often added to imaging cocktails and may function as a: triplet state quencher¹⁴⁰; ligand that forms a nonfluorescent adduct with a dye³²; reductant which can produce a long-lived, nonfluorescent radical species¹²⁵; deoxygenation agent by reacting with oxygen to form disulfides and water¹⁴¹; or perhaps a mixture of these or other yet unidentified roles. The introduction of an oxygen scavenging system can lower oxygen

concentrations substantially, but can lead to different outcomes. Oxygen is also a very efficient triplet state quencher which can yield higher fluorescence turnover rates (e.g., more photons per unit time) but which also leads to increased rates of photobleaching. For this reason, single molecule fluorescence studies often utilize an oxygen scavenger system in the imaging cocktail. Use of a reducing and oxidizing system (ROXS), such as the combination of methyl viologen and ascorbic acid together with an oxygen scavenging system, is a popular formulation to minimize photobleaching rates and blinking, although there are exceptions in which fluorophores still show substantial blinking under these conditions^{31,137}. Even the use of heavy water (D₂O) can impact photoswitching performance, such as for some oxazine and cyanine dyes which were recently reported to have a higher fluorescence quantum yield and/or higher photons per localization in heavy water as compared to normal water^{142,143}.

2.7 ADDITIONAL SUPER-RESOLUTION TECHNIQUES AND FLUORESCENT PROBES

In this article we have focused on fluorescent proteins and small organic fluorophores used in STED, RESOLFT/psSIM, or STORM/PALM. However, not all established techniques and not all fluorescent probes fit neatly into these categories. For instance, SOFI (super-resolution optical fluctuation imaging) uses higher order statistical analysis of fluctuating fluorescent signals in an image to generate super-resolution fluorescence images even without requiring the localization of individual fluorescent spots¹⁴⁴. Another technique, dubbed Bayesian localization microscopy, uses a more computation-intensive approach to model an entire set of fluorophore positions in a sample based on a series of measurements during which the fluorophore labels may blink or bleach¹⁴⁵. Additional classes of fluorescent probes have also been used in super-resolution microscopy, including quantum dots, fluorophores which reversibly bind to a target of interest, and organic fluorophores which turn on as a result of binding.

Quantum dots are intriguing fluorophores due to their high total photon yield and have been used in a number of super-resolution imaging papers. These go back to Lidke et al., 2005, whose work on independent component analysis with quantum dots presaged STORM/PALM type localization-based microscopy¹⁴⁶. Subsequent efforts have found application of quantum dots to imaging by RESOLFT, STORM/PALM, and SOFI although they have not found as frequent

use as fluorescent proteins or organic fluorophores^{144,147,148}. One concern regarding the use of core-shell quantum dots (which are also surface-passivated and functionalized) is their relatively large hydrodynamic radius (~20 nm), although this may potentially be improved by the use of smaller quantum dots in the future¹⁴⁹.

A subset of probes used for localization-based microscopy does not utilize photoactivation at all, but instead uses reversible binding with or without a chemical turn-on feature. For instance, Sharonov and Hochstrasser utilized the turn-on fluorescence property of the dye Nile red binding to the plasma membrane in their super-resolution method known as PAINT (points accumulation by imaging in nanoscale topography)³³. The approach has since been adapted to use the highly tunable binding/unbinding of fluorescently labeled DNA oligonucleotides to structures labeled with a complementary oligonucleotide, known as DNA-PAINT^{36,150}. DNA-PAINT has several attractive aspects, including being compatible with virtually any fluorophore, and the technique will likely find increased usage in coming years.

2.8 CONCLUSIONS

Over the past 5-10 years, super-resolution fluorescence microscopy has evolved from a small number of techniques used by a handful of specialist labs to a wide range of super-resolution techniques which can be found now at many major research institutions around the world. Much of the excitement over these tools relates to the ability to gain sharper glimpses of biological objects whose intrinsic size scales and density are often smaller than the diffraction limit of light, and an untold number of discoveries are hidden just beneath the diffraction limit. At this point in the field, even as the techniques push forward to new frontiers of spatial or temporal resolution or live cell capability, etc., it is more important than ever that new fluorophore developments include adequate characterization of the properties of the fluorophores so practitioners may evaluate whether or not the fluorophore is likely to be useful for their intended application. Hopefully, as super-resolution fluorescence microscopy matures into a workhorse tool for the biological imaging community, this will become standard practice for new reports on fluorophores or fluorophore properties. Only by these efforts can the best tools be placed into the hands of practitioners for widest impact.

2.9 ACKNOWLEDGEMENTS

This work was supported in part by the University of Washington and by a Burroughs-Wellcome Career Award at the Scientific Interface. The authors thank Dr. Aaron R. Halpern (University of Washington) for critical reading of this manuscript.

CHAPTER 3. EXTENDED-DEPTH 3D SUPER-RESOLUTION IMAGING USING PROBE REFRESH STORM (PRSTORM)

3.1 PREFACE

As discussed previously in **Chapter 1** and **Chapter 2**, at the moment there exists only one outstanding photoswitchable fluorophore for SMLM, Alexa 647. My initial work in the Vaughan lab was to characterize new organic fluorophores for SMLM synthesized primarily by Dr. Honglin Li with the goal of eventually finding better fluorophores for multicolor SMLM. However, after about a year focusing on this project I had created a set of assays and used them to characterize some of the new fluorophores, but the synthesis was slower than expected and few of the new fluorophores suited our purposes. At that point we decided to shift gears and I switched my focus to creating a method that would allow researchers to use Alexa 647 to image multiple channels.

I achieved this through the use of DNA-labeled antibodies which enabled fast and robust multichannel imaging. The technique was also nicely suited for tackling another challenge with SMLM, photobleaching of fluorophores in out of focus imaging planes which restricts imaging depth when trying to perform 3-dimensional SMLM. Together, this method, along with its two applications was published in *Biophysical Journal* in 2018.

I was heavily involved in this work at all stages of the project. I was responsible for the initial design and execution of the method, working out the best way to label antibodies with DNA, which sequences and lengths of DNA worked best, how much DNA can be labeled on the antibodies without impacting antigenicity and developing a simple but powerful quality control assay for quantifying the number of DNA per antibody. I was later joined in this project by fellow graduate student Mr. Marco Howard and Dr. Danying Lin, a visiting scientist from China. Together, the three of us were able to solidify a method for data collection and analysis to tackle both the multicolor and imaging depth challenges standard ways of performing SMLM face.

We all contributed to the outlining of the manuscript while I led the writing efforts. I collected most of data shown in the manuscript and supplementary information while Danying

and Marco were largely responsible for creating our data analysis pipeline. We all had a role in interpretation of our results.

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†Danying Lin; †**Lauren A. Gagnon**; †Marco D. Howard; Aaron R. Halpern; and Joshua C. Vaughan; “Extended-Depth 3D Super-Resolution Imaging Using Probe-Refresh STORM”, *Biophysical Journal* **114**, 1980-1987 (2018). Copyright 2018 Biophysical Society.

†Authors contributed equally to the work.

All material in this chapter has been reformatted to conform to the style of this thesis.

3.2 ABSTRACT

Single-molecule localization microscopy methods for super-resolution fluorescence microscopy, such as STORM (stochastic optical reconstruction microscopy), are generally limited to thin three-dimensional (3D) sections (≤ 600 nm) due to photobleaching of molecules outside the focal plane. Although multiple focal planes may be imaged prior to photobleaching by focusing progressively deeper within the sample, image quality is compromised in this approach because the total number of measurable localizations is divided between detection planes. Here we solve this problem, on fixed samples, by developing an imaging method, which we call probe refresh STORM (prSTORM), that allows bleached fluorophores to be straightforwardly replaced with non-bleached fluorophores. We accomplish this by immunostaining the sample with DNA-conjugated antibodies and then reading out their distribution using fluorescently-labeled DNA reporter oligonucleotides that can be fully replaced in successive rounds of imaging. We demonstrate that prSTORM can acquire 3D images over extended depths without sacrificing the density of localizations at any given plane. We also show that prSTORM can be adapted to obtain high-quality, 3D multichannel images with extended depth that would be challenging or impossible to achieve using established probe methods.

3.3 INTRODUCTION

Cells are dense, three-dimensional (3D) collections of molecules which are highly organized on the nanometer scale. Observing this nanoscale organization is crucial to understanding how cells function, but conventional fluorescence microscopy methods are unable to resolve objects closer together than ~ 250 nm due to the diffraction of light. To overcome this challenge, there has been a sustained interest in the development of super-resolution microscopy techniques that can maintain the high molecular specificity of fluorescence microscopy while allowing researchers to surpass the diffraction limit of visible light^{13,19,22,151}. Among these, single molecule localization microscopy (SMLM) techniques such as stochastic optical reconstruction microscopy (STORM) and photo-activated light microscopy (PALM) in their typical implementations achieve a spatial resolution of ~ 30 nm laterally and ~ 60 nm axially^{19,22}.

A range of approaches have been developed for 3D SMLM^{49,152–155}, but the easiest and most widespread of these uses a cylindrical lens to induce astigmatism in the detection path such that the ellipticity of single-molecule localizations can be used to determine the height of the molecules within the sample⁴⁹. However, in typical experiments where high numerical aperture oil immersion objective lenses are used to image into water based samples, substantial spherical aberration is incurred and the distal half of the focal depth in astigmatism imaging has notably poorer spatial resolution⁵¹. While the aberrations can be mitigated by using adaptive optics¹⁵⁶ or index matching solutions⁵¹, these are either costly or can compromise key focus lock systems that require refractive index contrast at the coverglass-water interface to function. Instead, a simple solution is to only accept localizations from the proximal half of the focal depth and to scan this high resolution portion of the focal plane through the sample in order to extend the depth range of imaging⁵¹. Unfortunately, this focal plane scanning process compromises the spatial resolution by dividing up the total localizations achievable among all focal planes (i.e., it lowers the localization density for any given plane), and it particularly degrades the resolution of focal planes imaged later in the process since they are partly photobleached during the imaging of other focal planes. There is thus a need for extended depth 3D SMLM methods which are easily implemented and can overcome the combined challenges of spherical aberration and photobleaching.

A second major challenge in SMLM is a lack of dyes across the visible spectrum which have excellent photoswitching for multichannel imaging. Methods using sequential rounds of staining and bleaching partially circumvent this problem by using the excellent fluorophore Alexa Fluor 647 (or its close structural relatives such as Cy5) multiple times, but full restaining has prohibitively long sample preparation times^{47,48,157}. A different sequential imaging strategy termed DNA-PAINT (points accumulation for imaging in nanoscale topography) generates SMLM data from transient binding and unbinding of fluorophore labeled oligonucleotides that are complementary to oligonucleotides decorating the specimen^{36,150}. Although DNA-PAINT does not require photoswitching and is therefore compatible with many fluorophores across the visible spectrum, its use of diffusing fluorescent probes creates high background signal unless low probe concentration and very low frame rates (~20 Hz) are used^{36,158,159}. More recently, new methods to streamline these processes utilizing DNA exchange probes have been introduced, making multichannel SMLM more accessible^{160,161}, although multichannel imaging continues to be a challenge for many SMLM applications.

Here we present a method to refresh fluorescent probes on fixed specimens to enable robust, multilayer 3D STORM over extended depths without compromised performance in any focal plane. We term this approach probe refresh STORM (prSTORM). We further demonstrate that the technique can be extended to multichannel imaging with uniform, high resolution in each channel, by multiple uses of the same fluorophore targeted to different structures. Our technique utilizes refreshable DNA probes to circumvent challenges faced when imaging in three dimensions with SMLM. Importantly, prSTORM can be implemented with common super-resolution microscopes with no modifications, and thus has the potential to benefit the whole community of SMLM researchers.

3.4 RESULTS

We developed prSTORM by utilizing custom antibody conjugates that are covalently bound by several (3 to 4) single-stranded DNA (ssDNA) oligonucleotides (Supplementary Table 3.1), each ~20 nucleotides (nt) in length^{162–164}. After immunostaining the specimen and treating it with a post-fixative (Supplementary Table 3.2), we exposed the specimen to a solution of pre-hybridized

adapter and fluorescently-labeled reporter oligonucleotides (Figure 3.1). The ~80 nt adapter strand contains a barcode recognition sequence (BRS) that is complementary to the ssDNA-antibody on the specimen as well as three repeats of a reporter docking sequence (RDS) that is complementary to the reporter strand (RS). Three repeats of the RDS were included in order to boost signal levels. This design allowed us to modify antibodies with several ~20 nt oligonucleotides rather than several ~60 nt oligonucleotides, in order to minimize any reductions of antibody affinity, while still targeting a sufficient number of fluorophores to each antibody molecule. The modular use of the adapter oligonucleotide was also convenient for multichannel prSTORM as described further, below.

Once labeled, the specimen is imaged in switching buffer (a deoxygenated solution containing a thiol; see methods) using astigmatism-based 3D STORM, the previous round's adapter/reporter oligonucleotides are completely removed through a brief exposure (<30 sec) to hot water (~90 °C), fresh (unbleached) probes are hybridized at room temperature (~25 °C, ~10 min), and the specimen is imaged at a new focal plane. The process is repeated for many cycles, with a period of ~30 minutes. Since our goal was to keep the higher resolution, ~300 nm layer of localizations beneath the focal plane, we advanced the focal plane by moving the objective ~300 nm per cycle (equivalent to ~210 nm in the cell due to focal shift⁵¹), taking care to ensure that each layer contains overlapping localizations along the axial dimension so that each slice can be properly aligned axially. After acquiring all layers, we analyzed the separate movies, computationally corrected for spherical-aberration-induced axial localization error⁵¹, extracted the ~300 nm sub-focal layer of localizations from each movie, and aligned all of the sections together to create a single multilayer 3D image. Overall, we found that the DNA labels on the secondary antibodies induced only mild spatial broadening of features compared to antibody labeling alone (Supplementary Figure 3.5), as has also been seen in other recent work using conventional and DNA-conjugated antibodies^{165,166}.

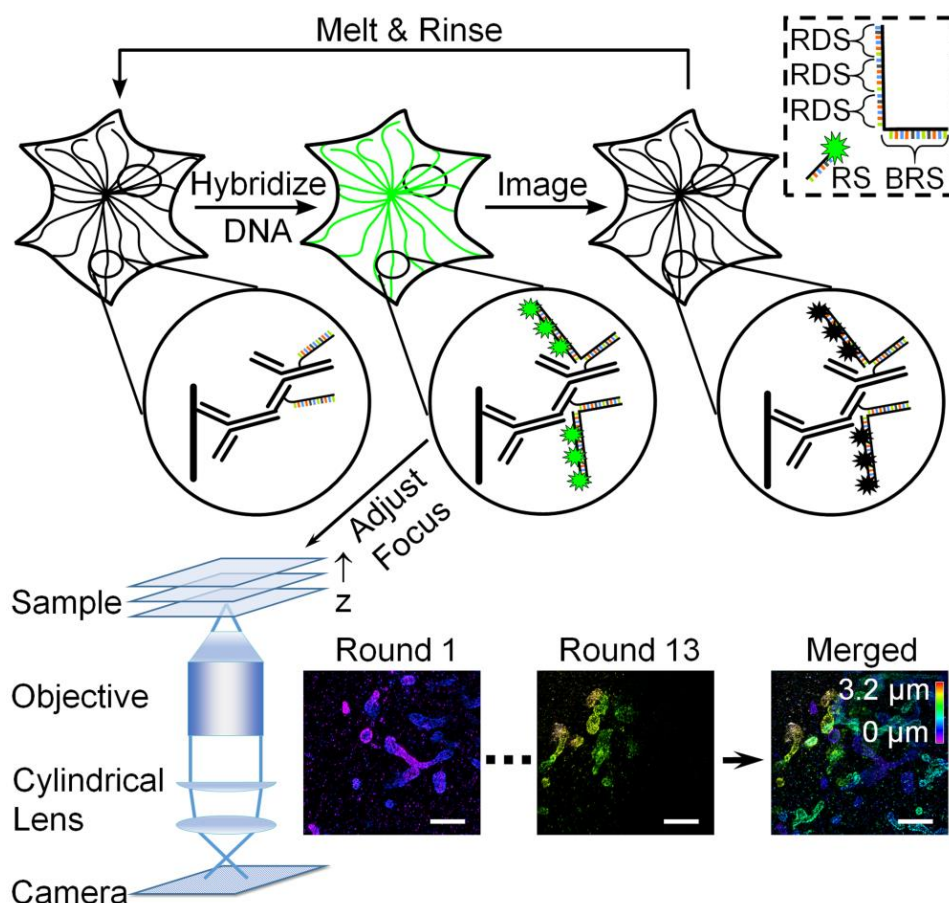


Figure 3.1. Workflow for prSTORM. A specimen immunostained with DNA-labeled secondary antibodies is exposed to a room temperature ($\sim 25^\circ\text{C}$) solution of pre-hybridized adapter and fluorescent reporter DNA, imaged by astigmatism 3D STORM, washed with hot water to remove photobleached adapter/reporter DNA, and a new focal plane is selected. This process is repeated many times in order to build up an extended depth 3D STORM image. The adapter oligonucleotide (inset, upper right) contains a barcode recognition sequence (BRS) which binds the antibody oligonucleotide as well as three reporter docking sequences (RDS) which can bind up to three fluorescently labeled reporter strands (RS). Scale bars: 2 μm .

3.4.1 Proof of concept

To demonstrate the ability of prSTORM to fully refresh the labels on the specimen without perturbation, we imaged the same specimen 10 times, each, with and without refreshing the probes (Figure 3.2a-c, Supplementary Figure 3.6a). The STORM images obtained with refreshed probes maintained the same number of localizations per round, while the non-refreshed specimen decreased substantially in each round due to photobleaching. We took efforts to make

the comparison as fair as possible by using 50,000 frames for each round of imaging, by omitting 405 nm activation that may be variable during the acquisition, and by replacing the switching buffer approximately every 15 minutes to limit potential interference caused by its decomposition over time¹⁶⁷. The difference between refreshed and non-refreshed is even more pronounced with longer movies (data not shown). In a second experiment, we found that even after 20 cycles of hybridization and melting at $\sim 90^\circ\text{C}$, there was negligible loss of signal and distortions were minimal when compared to the first round of imaging where the specimen had not been previously exposed to hot water (Supplementary Figure 3.7). Together, these show that our procedure is able to efficiently refresh fluorophores, in principle an unlimited number of times, without perturbing the nanoscale structure of the specimen.

We next performed multilayer 3D STORM with and without probe refresh steps on the same sample (Figure 3.2d-e, Supplementary Figure 3.6b). For this comparison, we used gradually increasing 405 nm laser activation to obtain the highest number of localizations possible from our sample before photobleaching. This “imaging to exhaustion” approach typically lasted $\sim 400,000$ frames per movie. In the probe refresh case, each of four layers was imaged to exhaustion with movies of $\sim 400,000$ frames, and we were able to reconstruct dense STORM images throughout the $\sim 1.2\ \mu\text{m}$ thickness of the specimen. In the non-refresh case, we divided the $\sim 400,000$ measurable frames among the four layers, and as expected the non-refresh sample was substantially sparser than in the refresh case, particularly for regions imaged later in the process since all layers are illuminated in each round of imaging, even those out of the focal plane. Thus, 3D prSTORM is able to substantially boost localization density over extended depths for multilayer imaging without suffering from loss of localization density due to photobleaching.

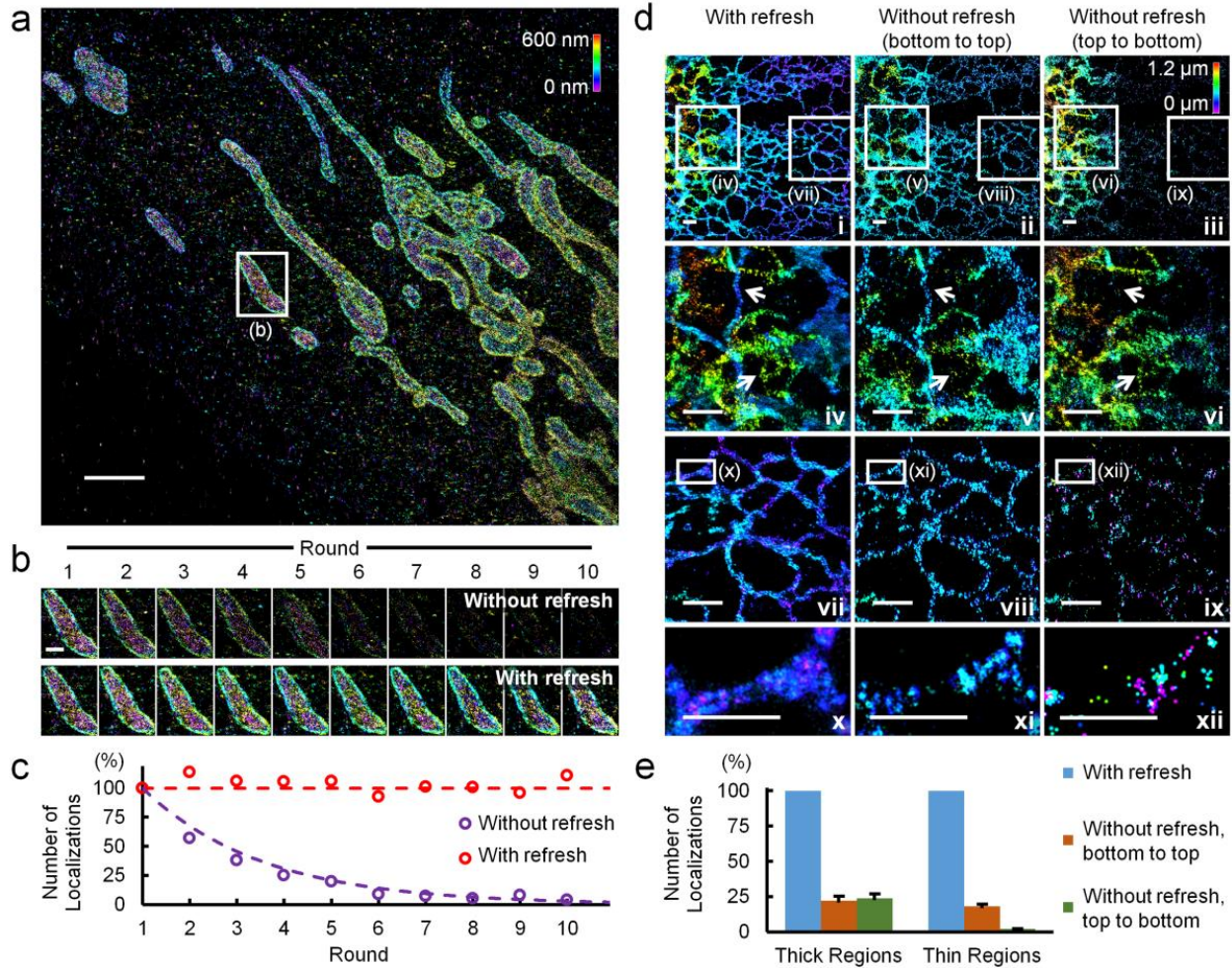


Figure 3.2. Improvement of single-molecule localization density due to probe refresh steps. **a)** 3D STORM image of in a BS-C-1 cell immunostained for TOM20 and imaged in a single round (see also Supplementary Table 3.2 for additional details). **b)** Images of the same boxed mitochondrion in **a** across 10 sequential rounds of imaging with and without probe refresh steps and **c)** quantification of localization density across many mitochondria in the same data set (see also Supplementary Figure 3.6a; the standard error of the mean was $<5\%$ of all mean values and was omitted from **c** for the sake of clarity). **d)** Four-layer extended-depth 3D STORM images, with and without probe refresh steps, of the endoplasmic reticulum (ER) in a BS-C-1 cell expressing sec61 β -GFP and immunostained for GFP. A higher number of localizations is achieved with probe refresh steps **i** than without, regardless of the scan direction **ii-iii**. Arrows in **iv-vi** indicate ER tubules at different depths within the cell which are only both visible when using probe refresh steps. Zoomed-in views **x-xii** show that hollowness of ER tubules is only evident when using probe refresh steps (**x**). The contrast for image panels in **d** was adjusted individually to allow visualization of details in the comparably sparse non-refresh images. **e)** Number of localizations (mean $\pm$ SEM) for thick and thin regions from **i-iii** showing that probe refresh steps increase the number of localizations (localization numbers were normalized to the refresh data; see also Supplementary Figure 3.6b). Scale bars: 2 μm (**a**), 1 μm (**d i-ix**), 500 nm (**b**, **d x-xii**).

3.4.2 *High density imaging of the endoplasmic reticulum*

We extended our use of prSTORM to 1.5 μm of the endoplasmic reticulum (ER), acquiring 200,000 frames per layer (Figure 3.3), as well as 3.2 μm of the mitochondrial network (Supplementary Figure 3.8). Without probe refresh steps we would be severely hindered by photobleaching, having only $\sim 40,000$ frames or less available per layer, which severely limits the achievable density of localizations per layer. The 3D view of the red box (Figure 3.3b) shows the thickest region of the ER and we see from the x-z and y-z slices that with probe refresh steps we obtain even axial resolution in all layers. We were also able to resolve holes in the “sheet-like” structures (Figure 3.3c-d) which are consistent with a recent report that suggests ER sheets are actually dense matrices of tubules¹⁶⁸.

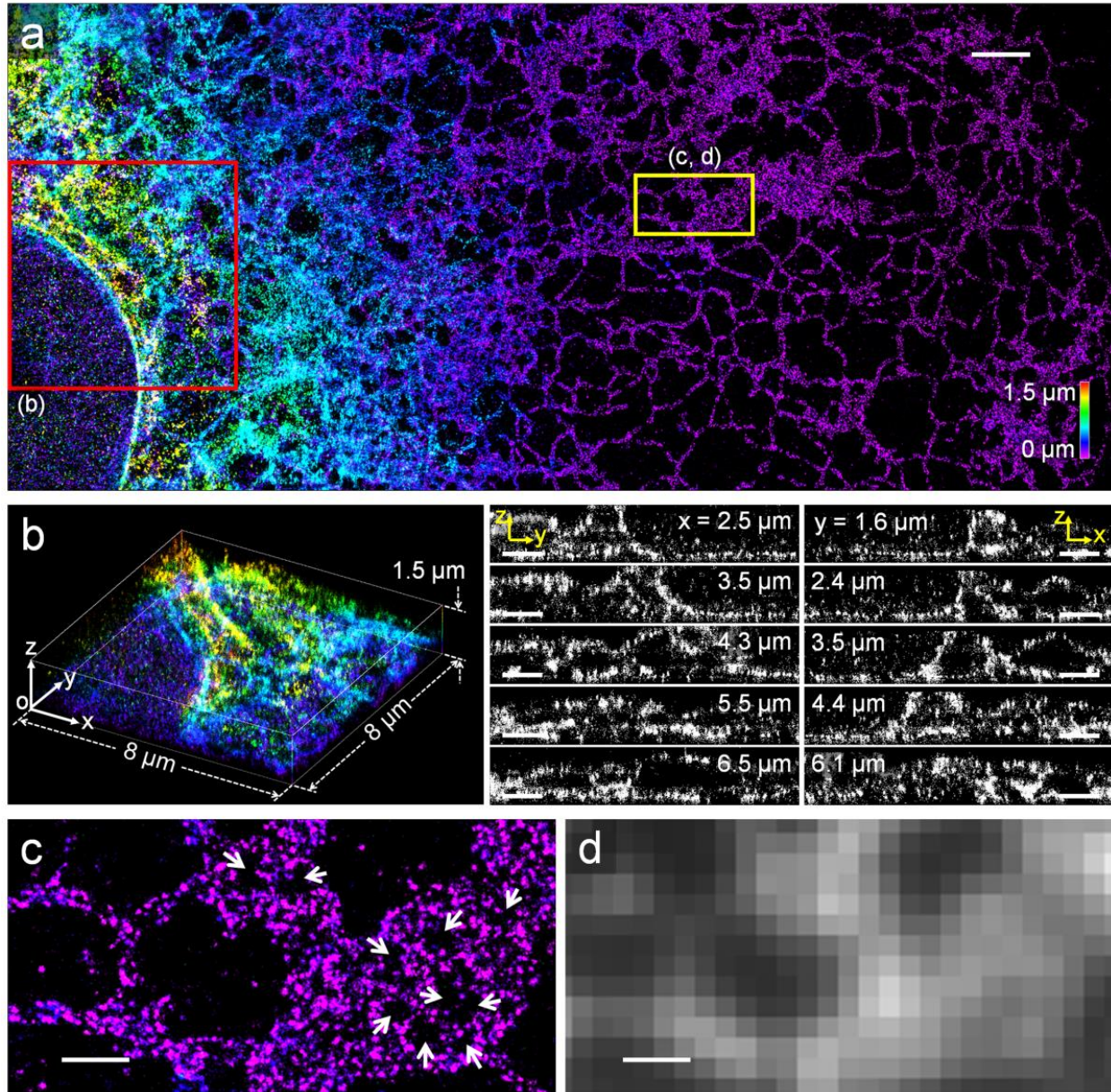


Figure 3.3. Extended depth 3D prSTORM image of the endoplasmic reticulum (ER) in COS-7 cells expressing sec61 β -GFP and immunostained for GFP (see also Supplementary Table 3.2 for additional details). **a)** x-y projection colored in z. **b)** 3D rendered perspective view (left panel) of red box in **a**, with associated y-z sections (middle column), and x-z sections (right column). Section thickness: 100 nm. **c)** Zoomed-in view of yellow box in **a**. Arrows indicate gaps in the ER tubule matrices. **d)** Conventional fluorescence image corresponding to **c**. Scale bars: 2 μ m (**a**), 1 μ m (**b**), 500 nm (**c-d**). Positions are indicated relative to the origin "o" shown in panel **b**.

3.4.3 *Multichannel imaging with prSTORM*

We found that prSTORM was easily adapted for use with multichannel imaging (see schematic in Supplementary Figure 3.9). To do so, we prepared three different secondary antibodies, each functionalized with a unique ssDNA oligonucleotide, and we purchased three different ~80 nt adapter nucleotides with orthogonal BRSs that can hybridize with the three antibody ssDNA oligonucleotides. By using the same RDS on each of these three adapter oligonucleotides, we were able to use the same fluorescently labeled reporter oligonucleotide for all three adapters. This modularity simplified our approach and only required the use of a single reporter oligonucleotide that was labeled with the excellent STORM fluorophore Alexa Fluor 647. Thus, each channel would be imaged with the superior fluorophore Alexa Fluor 647 rather than using, for instance, multiple spectrally independent fluorophores which inevitably have one excellent fluorophore (typically Alexa Fluor 647 is used in the channel with the most demanding structure to image at high resolution) and one or more fluorophores with notably poorer performance⁴³. The easily refreshable probes also allowed us to label all three targets simultaneously prior to sequential labeling and imaging of each structure, which simplified image registration among multiple channels.

We demonstrate multichannel/multilayer 3D prSTORM imaging of mitochondria, microtubules, and the ER by recording a 200,000 frame movie for each structure in each layer, with 405 nm activation, spanning an axial depth of ~800 nm (Figure 3.4). A high density of localizations was achieved on each structure over the depth of the specimen, giving high resolution images (Figure 3.4a-b) and we saw little evidence of crosstalk between the channels (Figure 3.4c). In a series of control experiments we found that our washing procedure was able to remove ≥98% of fluorescent signal from the specimen (data not shown) and we observed low crosstalk (<2-5%) between any of the channels (Supplementary Figure 3.10). We observed that mitochondria generally localized to regions of the cell with “sheet-like” ER structures and are mostly absent from regions with only sparse ER tubules. In both the x-z and y-z slices we observe hollow mitochondria. Additionally, we are able to ascertain axial relationships among structures which are not easily discernable from the 2D projections, such as microtubules crossing just above and below ER sheets (Figure 3.4 b-c).

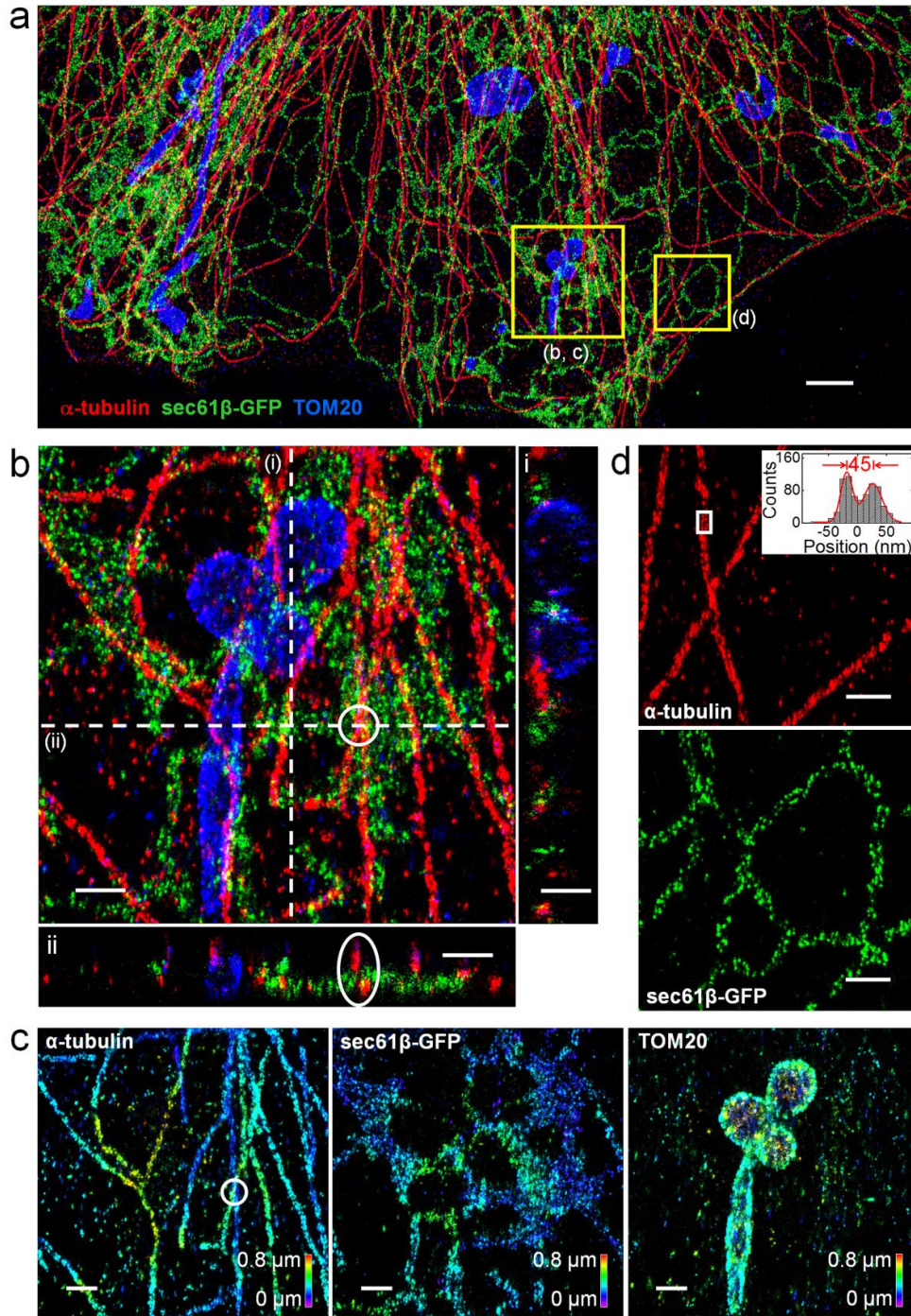


Figure 3.4. Three-channel, extended depth 3D prSTORM images of microtubules, mitochondria, and the endoplasmic reticulum (ER) in COS-7 cells expressing sec61 β -GFP that were immunostained for α -tubulin, TOM20, and GFP (see also Supplementary Table 3.2 for additional details). **a)** Three-color x-y projection. **b)** Zoomed-in view of box in **a**, with orthogonal views of the dashed cross (100 nm section thicknesses). **c)** x-y projections colored in z for each structure (tubulin, ER, and mitochondria, respectively). White circle indicates the same sites marked in **b**. **d)** Zoomed-in view of box in **a**. Inset shows cross-sectional profile of the tubulin channel. Scale bars: 2 μ m (**a**), 500 nm (**b-d**).

3.5 DISCUSSION

We have described and demonstrated prSTORM, an approach for extended depth single-molecule localization microscopy which uses probe refresh steps to circumvent limits otherwise caused by photobleaching of fluorophores on fixed specimens. Our procedure for refreshing the probes is simple to implement and many cycles of melting, hybridization, and washing produced little to no detectable distortions on the sample. We showed that prSTORM extends the depth achievable by 3D STORM without sacrificing the density of localizations. Although we used astigmatism-based axial localization here, together with the collection of only the proximal half of localizations that are of higher spatial resolution (~ 300 nm range) to measure specimens up to $3.2\text{ }\mu\text{m}$ thick, we note that prSTORM can extend the depth achievable with any axial localization technique¹⁶⁹ by eliminating limitations previously imposed by photobleaching. We also showed that prSTORM extends straightforwardly to multichannel imaging over an extended depth range with excellent performance in each of the channels and low crosstalk. The application of prSTORM to the imaging of thicker specimens, including tissue sections, may face additional challenges, including more prominent spherical aberration, poorer accessibility of antibodies or oligonucleotides, and a higher background signal. As a result, some changes to the protocol or compromise in resolution or speed may be necessary.

3.5.1 *Comparison between prSTORM and DNA-PAINT*

DNA-PAINT uses transient binding of diffusing probes and can also be performed for very long periods without photobleaching, so it is worth comparing its performance with that of prSTORM. In DNA-PAINT, the binding rate of reporter oligonucleotides is diffusion-limited and is controlled primarily by adjusting their concentration. Because the freely diffusing reporter oligonucleotides contribute to substantial background that can obscure the signal of a single, bound reporter oligo, the reporter oligonucleotides are typically used at relatively low concentrations. As a result, frame rates for DNA-PAINT are generally quite low (~ 20 Hz), such that in a 30 minute period, a maximum of $\sim 36,000$ frames could be recorded. In contrast, photoswitching with Alexa Fluor 647 can be performed with frame rates of 100-1000 Hz. Here, with prSTORM, we recorded $\sim 200,000$

frames in ~17 minutes (at 200 Hz) with an additional ~11 minutes for melting, hybridizing probes, and washing, for a total of <30 min/cycle for 5-6 times more frames. Since the maximum information content attainable is proportional to the number of frames acquired, 200,000 frames can provide more than 5 times as many localizations than 36,000 frames. Scientific CMOS (complementary metal-oxide-semiconductor) cameras are now routinely used for ~1000 Hz STORM, and would allow the prSTORM cycle time to be shortened to ~15 min for 200,000 frame movies, or would, for instance, allow 200,000 frame movies to be recorded in each of several regions of interest within 30 min. DNA-PAINT with a confocal microscope of whole cells has recently been reported, but only for a modest number of frames per focal plane (10,000-30,000), presumably due to the low frame acquisition rate of ~5 Hz¹⁵⁹. Additionally, FRET-PAINT based on Förster resonance energy transfer (FRET) probes has been used to decrease the fluorescent background and to allow a faster frame acquisition rate than standard DNA-PAINT (≤ 70 Hz compared to ~20 Hz), but with some compromise of the resulting image quality¹⁷⁰. Overall, prSTORM can much more rapidly achieve a high density readout than DNA-PAINT. However, there may be situations where the potential high photon/localization capability of DNA-PAINT provides superior results, particularly for relatively sparse samples when very high resolution localization is desired¹⁷¹.

3.5.2 *Comparing prSTORM to other sequential multichannel imaging methods*

Beyond DNA-PAINT, other methods for sequential multichannel imaging have been demonstrated. In methods based on cyclic immunofluorescence, a specimen is immunolabeled against one protein using Alexa Fluor 647, imaged, and the fluorescent signal is removed by photobleaching of fluorophores, chemical bleaching of fluorophores, or removal of the antibodies by salt, heat, etc.^{47,48,157}. After quenching the fluorescence, the specimen is removed from the stage, immunostained against second protein, and imaged. Each round of imaging takes at least an hour because the specimen must be incubated with primary and secondary antibodies, blocked, and quenched again^{47,48}. prSTORM and other DNA-barcoding based methods¹⁷² present a distinct advantage because the specimen can be completely labeled with all antibodies at the

benchtop and then these can be read out sequentially using rapid DNA hybridization and melting steps performed on the microscope.

3.5.3 *Drawbacks of prSTORM and proposed improvement strategies*

While the DNA-conjugated antibodies enable prSTORM, they also face some drawbacks. Preparation of the antibodies, while documented well^{160,161,173,174}, takes a few days and time to gain familiarity with the reagents and procedures. The DNA antibody labels also modestly broaden features^{165,166} slightly beyond the use of secondary antibody labeling alone (Supplementary Figure 3.5). However, this DNA-induced broadening could be reduced in the future through some combination of the following: using shorter hybridizing sequences (~15 nt); using only two repeats of the reporter docking sequence; using 3'-labeled reporter oligonucleotides (with adapters); omitting use of the adapters and using 5'-labeled reporter oligonucleotides that bind directly to the antibody-linked oligonucleotide; or conjugating DNA to primary antibodies. We also note that, like other immunofluorescence techniques involving intracellular targets, prSTORM is not compatible with live cell imaging.

In terms of hardware, prSTORM utilizes on-stage exchanges of solutions to refresh probes and thereby circumvents problems due to photobleaching. In this work we performed these solution exchanges manually using a simple and inexpensive apparatus assembled from commercially available cell culture channels and a fixed-height aspirator that is very easy to set up with components already available in most labs (Supplementary Figure 3.11). The few necessary components can be easily brought to a central imaging facility or set up in labs with a STORM microscope. Although it is not necessary, the entire process could be automated using computer-controlled focal plane scanning and a computer-controlled perfusion system for solution exchanges, etc., in order to boost throughput and to reduce human labor.

3.6 CONCLUSION

We have developed and characterized prSTORM which permits extended depth 3D super-resolution microscopy without incurring limitations resulting from photobleaching. We showed that prSTORM enables high resolution imaging throughout multiple layers of a specimen without

loss of resolution due to reduced localization density and we also show that prSTORM can be easily extended to multiple channels with low crosstalk. The probe refresh method would potentially also benefit other 3D SMLM techniques, such as 4Pi interferometry, as well as stimulated emission depletion (STED) microscopy or structured illumination microscopy (SIM) so that a superior fluorophore or wavelength range could be used for multiple channels over extended depths.

3.7 ACKNOWLEDGMENTS

This work is partially supported by the University of Washington (J.C.V.), a Burroughs-Wellcome Career Award at the Scientific Interface (J.C.V.), NIH grant R01MH115767 (J.C.V), an NSF Graduate Research Fellowship DGE-1256082 (M.D.H.), the National Basic Research Program of China (No. 2015CB352005, D.L.), and the National Natural Science Foundation of China (No. 61775144, D.L.). The authors would like to thank Jonathan Perr (University of Washington, Seattle, WA) for his help with antibody labeling, Linda Wordeman (University of Washington, Seattle, WA) for access to a Lonza 4D X nucleofector system, and Tom Rapoport (Harvard Medical School, Boston, MA) for the Sec61 β -GFP plasmid. The authors declare no competing financial interests.

3.8 MATERIALS AND METHODS

3.8.1 *Reagents*

Alexa Fluor 647 N-hydroxysuccinimide (NHS) ester; Alexa Fluor 750 NHS ester; 10 $\times$ phosphate buffered saline (PBS); sodium chloride (NaCl); ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt (EGTA); magnesium chloride hexahydrate (MgCl₂); saponin; d-glucose anhydrous; 2-(N-morpholino) (MES) buffered saline packs; hydrochloric acid (HCl); sodium hydroxide (NaOH); Zeba spin desalting columns (100 kDa molecular weight cut off); disodium hydrogen phosphate (Na₂HPO₄); and tris base were purchased from Thermo Fisher Scientific (Waltham, MA). Sodium bicarbonate; dimethyl sulfoxide (DMSO); dimethylformamide (DMF); triton X-100; piperazine-N,N'-bis(2-ethanesulfonic acid) sesquisodium salt (PIPES); 2-

hydrazinopyridine dihydrochloride (2-HP); sodium borohydride (NaBH_4); tRNA from baker's yeast; 2-mercaptoethanol (BME); glucose oxidase from *Asperigillus niger*; catalase; and Ultra-0.5 mL centrifugal filters (100 kDa molecular weight cut off) were purchased from MilliporeSigma (St. Louis, MO). N-succinimidyle 4-formylbenzoate (SFB) was purchased from TCI America (Portland, OR). Succinimidyl 6-hydrazinonicotinate acetone hydrazone (SANH) was purchased from Molecular Targeting Technologies (West Chester, PA). 10x Turbolink™ Catalyst Buffer was purchased from Solulink (San Diego, CA). Bovine serum albumin (BSA) was purchased from Rockland Immunochemicals (Limerick, PA). Aqueous solutions of 32% paraformaldehyde and 50% glutaraldehyde were purchased from Electron Microscopy Sciences (Hatfield, PA). Illustra Nap-5 columns, Sephadex G-25; and Hyclone Fetal Bovine Serum (FBS) were purchased from GE Healthcare (Chicago, IL). Vivaspin 500 centrifugal concentrators (5 kDa molecular weight cut off) were purchased from Vivaproducts (Littleton, MA). Commercially available mouse monoclonal anti-GFP epitope (1:200; IgG_{2a}; clone B-2; Santa Cruz Biotechnology (Dallas, Tx)); rabbit polyclonal anti-Tom20 epitope (1:200; clone FL-145; Santa Cruz Biotechnology); and rat monoclonal anti-alpha tubulin epitope (1:500; clone YL1/2; Thermo Fisher Scientific) were used for immunohistochemistry. Donkey anti-rat IgG (712-005-153); donkey anti-rabbit IgG (711-005-152); and donkey anti-mouse (715-005-151) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Microscope objective lens immersion oil (Laser liquid oil, code 5610) was purchased from Cargille Laboratories (Cedar Grove, NJ). Roswell Park Memorial Institute (RPMI) Medium 1640 [+] L-glutamine; Minimal Essential Medium (MEM) non-essential amino acids (NEAA); penicillin streptomycin; trypsin ethylenediaminetetraacetic acid (EDTA); and Dulbecco's Modified Eagle Medium (DMEM) [+] 4.5 g/L D-glucose, [+] L-glutamine, and [+] 110 mg/L sodium pyruvate were purchased from Gibco via Thermo Fisher Scientific.

3.8.2 *Secondary antibody conjugation to Alexa Fluor 647 for standard immunohistochemistry (no DNA)*

Secondary antibodies for standard immunohistochemistry were labeled with Alexa Fluor 647 NHS ester at a labeling efficiency of 3-4 dyes per antibody according to the following procedure. 80 µL of secondary antibodies (~1.3 mg/mL) were incubated with 10 µL 1 M aqueous sodium

bicarbonate (pH ~8.3) and 4-6 μg of Alexa Fluor 647 NHS ester at room temperature for 45 minutes. Concentrated dye stocks (~100 $\mu\text{g}/\mu\text{L}$) were prepared in anhydrous DMSO and stored at -20 °C and diluted into sub-aliquots for the labeling reactions. During the reaction we equilibrated a Nap-5 column with ~10 mL of PBS. After 45 minutes, we loaded the antibody mixture onto the column (~100 μL), we allowed the antibody mixture volume to sink into the frit, flowed 600 μL of PBS through the column (discarding the flow through), added 300 μL of PBS to the column and collected the 300 μL of eluate, which contained the Alexa Fluor 647 modified antibody. We then characterized the eluate using a UV-Vis spectrometer. Antibody concentration [IgG] and labeling ratio were determined using equations 3.1-3.23.1, where A_{280} is absorbance at 280 nm, $CF_{\text{dye},280}$ is the dye 280 nm correction factor (0.03 for Alexa Fluor 647), A_{dye} is the dye peak absorbance (at 650 nm for Alexa Fluor 647), $\epsilon_{\text{IgG}}=200,000 \text{ M}^{-1}\text{cm}^{-1}$ is the IgG extinction coefficient at 280 nm, $L=1 \text{ cm}$ is cuvette path length, and ϵ_{dye} is the dye peak extinction coefficient ($\epsilon_{\text{dye}}=239,000 \text{ M}^{-1}\text{cm}^{-1}$ at 650 nm for Alexa Fluor 647). We typically achieved an antibody concentration of ~0.0013 mM (0.20 mg/mL).

$$[\text{IgG}] = \frac{A_{280} - (CF_{\text{dye},280} \times A_{\text{dye}})}{\epsilon_{\text{IgG}} \times L} \quad (3.1)$$

$$d/p = \frac{A_{\text{peak, dye}} \times \epsilon_{\text{IgG}}}{\epsilon_{\text{dye}} (A_{280} - (CF_{\text{dye},280} \times A_{\text{dye}}))} \quad (3.2)$$

3.8.3 Preparation of DNA-antibody conjugates

Secondary antibodies were conjugated to amine-modified DNA sequences using a modified version of a previously published protocol^{173,175} which is also described in detail, below. Overall the procedure consists of functionalizing amino-modified DNA with SFB, functionalizing antibodies with SANH, and coupling the SFB and SANH groups together to produce DNA-antibody conjugates.

Preparation of buffers. 250 mL of Buffer A (150 mM NaCl, 100 mM Na_2HPO_4 , pH 7.4) was prepared by combining ~2.2 g NaCl and ~3.5 g Na_2HPO_4 and deionized water to a total volume of 250 mL.

Buffer C was prepared using the same recipe and adjusted to pH 6.0. 2× MES, pH 5.0 was prepared by dissolving one packet MES buffered saline in deionized water to a total volume of 250 mL. The pH of buffers were adjusted to the indicated pH values using 10 M HCl or 10 M NaOH.

Modification of amino-oligonucleotide with SFB. DNA sequences containing 5' amine modifications (5' amino C6) were purchased from IDT (Integrated DNA Technologies) in 0.1-1 μ mol synthesis scale. Before opening, IDT tubes were centrifuged briefly to ensure DNA pellet is positioned at the bottom of the tube. After centrifugation, the DNA was dissolved in water at a concentration of 10 mM (10 nmol/ μ L). This stock was stored at -20 °C for future use. For a 200 nmol synthesis, 20 μ L of stock DNA was diluted with 80 μ L of Buffer A, yielding a final concentration of 2 mM DNA in Buffer A. ~1 mg SFB was suspended in 40 μ L anhydrous DMSO (71.5 mM). 40 μ L of the 71.5 mM SFB solution per 200 nmol DNA was suspended in Buffer A.

The solution was vortexed gently and allowed to react at room temperature for 2 hours. We removed excess SFB from the DNA using a Vivaspin 500 centrifugal filter by adding the reaction to the spin filter and bringing the total volume to ~500 μ L using Buffer C. The reaction was centrifuged for 10 minutes at 13000 g. The flow-through was discarded and Buffer C was added to a volume of ~500 μ L before centrifuging for 10 minutes at 13000 g. This process was repeated 3 additional times. After centrifuging we pipetted out the concentrate (~80 μ L) and brought the total volume up to 100 μ L using Buffer C, rinsing the filter with the volume of Buffer C we added to the concentrate. Solutions of SFB-modified DNA in Buffer C were stored at 4 °C for up to a year.

We quantified the amount of SFB-modified DNA by measuring the Molar Substitution Ratio (*MSR*). To do so, we prepared a working solution of 0.5 mM 2-HP in 0.1 M MES buffer, pH 5.0. This working solution is stable when stored at 4 °C and protected from light. Next, we prepared a 2-HP blank solution by adding 2 μ L water to 48 μ L 2-HP working solution and prepared a SFB-oligo *MSR* solution by adding 2 μ L of purified oligo to 48 μ L of 2-HP working solution. We then vortexed and incubated these two solutions at 37 °C for 1 hour. After incubating, there was often some condensation on the inner caps of the tubes which we collected by centrifuging the tubes for ~15 seconds at 15000 g. Next, we added 450 μ L water to each tube and briefly vortexed;

this dilution typically brought its absorption to <1 on our UV-vis but it may need to be adjusted depending on the reaction. The reaction of 2-HP with the modified DNA sample forms a hydrazone bond with absorbance at 354 nm (molar extinction coefficient = 28,500 M⁻¹cm⁻¹). We used a UV-vis to quantify the substitution ratios, blanking the instrument with the prepared 2-HP blank solution. The concentrations of modified oligo and total oligo in solution we calculated using the 354 and 260 nm absorption peaks, respectively. Molar extinction coefficients for DNA sequences are provided by IDT. The ratio of these two concentrations yields the *MSR* as shown in equation 3.3, where A_{260} is the absorbance at 260 nm, A_{354} is the absorbance at 354 nm, and ϵ_{oligo} is the extinction coefficient of the oligo. We typically achieve *MSR* values of 0.75 and modified DNA concentrations of ~1 mM using this method. The concentration of modified DNA stock is multiplied by 250 in equation 3.4 to compensate for the two dilution steps.

$$MSR = \frac{[\text{Modified oligo}]}{[\text{Total oligo}]} = \frac{\left[\frac{A_{354}}{28,500 \frac{1}{\text{M} \times \text{cm}}} \right]}{\left[\frac{A_{260}}{\epsilon_{\text{oligo}}} \right]} \quad (3.3)$$

$$[\text{Modified DNA}]_{\text{Stock}} = MSR \times \frac{A_{260}}{\epsilon_{\text{oligo}} \times L} \times 250 \quad (3.4)$$

Modification of antibodies with Alexa Fluor 750. Prior to modifying antibodies with SANH, we found it useful to label them with Alexa Fluor 750 at a *d/p* ratio of 0.5-1 in order to quantify the final DNA-antibody conjugate labeling ratio which may otherwise be confounded by overlapping peaks due to antibody absorption at ~280 nm and DNA absorption at ~260 nm. We used a protocol similar to modification of antibodies with Alexa Fluor 647, described above, but with changes described here. We added ~3 µg of Alexa Fluor 750 NHS ester from a concentrated DMSO stock and 50 µL of freshly made 1 M aqueous sodium bicarbonate (pH ~8.3) to 200 µL of antibody (~1.3 mg/mL antibody in PBS), and incubated the reaction at room temperature (23 °C) for 45 minutes. During the reaction, we equilibrated a Nap-5 column by flowing through ~10 mL of Buffer A. After 45 minutes, we loaded the antibody mixture onto the column (~250 µL), we allowed the antibody mixture volume to sink into the frit, flowed 450 µL of Buffer A through the column (discarding the flow through), added 300 µL of Buffer A to the column and collected the

300 μL of eluate, which contained the Alexa Fluor 750 modified antibody. We characterized the number of Alexa Fluor 750 dyes per IgG (d/p) using equation 3.2 with suitable parameters for Alexa Fluor 750 ($CF_{\text{dye},280}=0.04$, A_{dye} is for the absorbance at 750 nm, and $\epsilon_{\text{dye}}=290,000 \text{ M}^{-1}\text{cm}^{-1}$). We typically achieved $d/p=0.5$ -1 and an antibody concentration of $\sim 0.0025 \text{ mM}$ (0.37 mg/mL).

Modification of antibody with SANH. We dissolved 1 mg of SANH in 350 μL of DMF (9.8 mM) and stored in a desiccator at room temperature (23 $^{\circ}\text{C}$) for up to 2 weeks. We added $\sim 7.5 \mu\text{L}$ of the SANH solution (see equations 3.5 and 3.6) to 300 μL of Alexa Fluor 750 modified antibody stored in Buffer A, vortexed, and incubated at room temperature for 2 hours. The molar excess of SANH over IgG can be adjusted to tune the number of DNA strands that bind to the IgG using equations 5 and 6. At a 100 molar excess of SANH ($\sim 7.5 \mu\text{L}$ SANH stock) we typically achieved 3-4 DNA/IgG. We found that higher DNA/IgG ratios could substantially disrupt antibody affinity.

$$[\text{SANH}]_{\text{final}} = [\text{IgG}] \times \text{Molar Excess} \quad (3.5)$$

$$\text{Volume}_{\text{SANH}} = \frac{[\text{SANH}]_{\text{final}} \times \text{Volume}_{\text{IgG}}}{9.8 \text{ mM}} \quad (3.6)$$

Ten minutes before the reaction completed, we prepared Zeba spin columns. The column's bottom closure was removed and the cap was loosened. We placed the column in a microcentrifuge tube and centrifuged at 1500 g for 1 min to remove the storage solution. The flow-through was discarded and we placed the column back into the collection tube, added 300 μL of Buffer C on top of the resin, and centrifuged the column again. The flow-through was discarded and the process repeated two additional times before placing the column in a clean microcentrifuge tube. We then applied the SANH-IgG reaction solution to the column and centrifuged at 1500 g for 2 minutes. We saved the flow-through and discarded the column.

Conjugation of SFB modified oligo to SANH modified antibody. We determined the volume of SFB-oligo to add to SANH-IgG using equations 3.7 and 3.8. SANH-IgG concentration can be assumed

to be the concentration determined after modification with Alexa Fluor 750. We typically use a 15-100 fold molar excess of SFB to SANH-IgG depending on the DNA sequence/antibody pairs being used. We found reaction efficiency to vary among sequences/antibody pairs.

$$[\text{SFB-oligo}]_{\text{final}} = [\text{SANH-IgG}] \times \text{Molar Excess} \quad (3.7)$$

$$\text{Volume}_{\text{SFB-oligo}} = \frac{[\text{SFB-oligo}]_{\text{final}} \times \text{Volume}_{\text{SANH-IgG}}}{[\text{Modified DNA}]_{\text{Stock}}} \quad (3.8)$$

We added the appropriate volume of SFB-oligo to SANH-IgG along with 1/9th of the total volume of Turbolink Catalyst Buffer (10×), vortexed the solution, and incubated at room temperature for 2 hours at room temperature (~23 °C) or overnight at 4 °C. For purification, we placed an Amicon Ultra 0.5 mL filter in a collection tube and applied the reaction solution to the filter. We added 1× PBS to the filter to make the total volume ~500 µL and centrifuged for 5 minutes at 14000 g. The flow-through was discarded and 1× PBS was added to bringing the total volume back up to ~500 µL and centrifuge again for 5 minutes at 14000 g. We repeated this step an additional three times. Next, we pipetted out the concentrate and measured its volume. We brought the total volume up to 100 µL using PBS by first rinsing the filter with the volume to be added to the concentrate. We used the UV-vis to measure the 354 and 750 nm absorbance peaks and determined the DNA/IgG labeling ratio and IgG concentration using equations 3.9-3.11, where A_{750} is absorbance at 750 nm, $29,000 \text{ M}^{-1}\text{cm}^{-1}$ is the extinction coefficient of the hydrazone bond between DNA and IgG, $290,000 \text{ M}^{-1}\text{cm}^{-1}$ is the extinction coefficient of Alexa Fluor 750, and d/p is dye to protein ratio for Alexa Fluor 750.

$$[\text{DNA-IgG}] = \frac{A_{354}}{29,000 \frac{1}{\text{M} \times \text{cm}} \times L} \quad (3.9)$$

$$[\text{IgG}] = \frac{A_{750}}{290,000 \frac{1}{\text{M} \times \text{cm}} \times L \times d/p} \quad (3.10)$$

$$\frac{\text{DNA}}{\text{IgG}} = \frac{[\text{DNA-IgG}]}{[\text{IgG}]} \quad (3.11)$$

3.8.4 Cell culture and transfection

BS-C-1 cells (ATTC CCL-26) were grown in RPMI supplemented with 1x NEAA, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% FBS. COS-7 cells (ATCC CRL-1651) were grown in

DMEM supplemented with 1x NEAA, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% FBS. In some cases, COS-7 cells were transfected with Sec61β-GFP fusion plasmid using a Lonza 4D X Nucleofector system. First, COS-7 cells were grown to approximately 90% confluency and approximately 2-4 million cells were harvested using 0.25% Trypsin EDTA. The cells were mixed with 18 µL of SE cell supplement and 82 µL of SE nucleofection solution from Lonza and 2 µg/million cells of with Sec61β-GFP fusion plasmid. Then the cells were placed in the Lonza 4D nucleofector system and transfected using the DS-120 pulse code. Cells were then recounted and seeded into chambers with ~30,000 cells/well for ibidi µ-Slide 8 well glass bottom chambers or ~11,000 cells/channel for ibidi µ-Slide VI 0.4 glass bottom flow channels (~11,000 cells/channel), and incubated overnight.

3.8.5 *Cell fixation*

All samples were fixed at 37 °C for 10 minutes in PFA/GA fixation solution containing 100 mM aqueous PIPES buffer pH 7, 1 mM EGTA, 1 mM MgCl₂, 3.2% paraformaldehyde, 0.1% glutaraldehyde, then reduced for 10 minutes with fresh, 10 mM aqueous sodium borohydride, and rinsed with PBS for 5 minutes. Prior to fixation, some specimens were subjected to a ~20 second extraction step with a solution of 100 mM aqueous PIPES buffer pH 7, 1 mM EGTA, 1 mM MgCl₂, which contained either 0.025% saponin or 0.5% triton X-100; see Supplementary Table 3.2 for additional details.

3.8.6 *Immunostaining*

All samples were incubated at room temperature for 30 minutes in block/perm solution containing PBS, 3% BSA, and 0.5% triton X-100. All samples were incubated for 30 minutes to overnight in block/perm solution containing primary antibodies. Standard immunohistochemistry samples containing no DNA were incubated for 30 minutes in block/perm solution containing secondary antibodies labeled with Alexa Fluor 647. DNA samples were incubated for 30 minutes with secondary antibody-DNA conjugates in DNA block/perm solution containing PBS, 3% BSA, 0.5% triton X-100 and 1 mg/mL yeast tRNA, and then rinsed for 5 minutes with PBS. All samples were post-fixed for 10 min in a solution containing PBS, and 0.25%

glutaraldehyde. Adapter and reporter ssDNA were pre-hybridized for 10 min in DNA block/perm. Samples were incubated with pre-hybridized adapter/reporter solution at room temperature or 37 °C for 5-10 minutes followed by a 3 mL rinse with PBS. See Supplementary Table 3.2 for additional details.

3.8.7 *STORM setup and imaging*

Imaging was performed on a homebuilt STORM system constructed on an inverted microscope (Eclipse Ti-U, Nikon), equipped with a 100X 1.45NA oil objective (CFI Plan Apo λ , Nikon), a motorized stage (MS-2000 Flat-Top, Applied Scientific Instrumentation) and a custom-built focus lock module based on a piezoelectric objective lens nano-positioner (Nano-F100S, Mad City Labs) and an infrared laser diode (LP940-SF30, Thorlabs). The 647 nm illumination laser (2RU-VFL-P, MPB Communication) was controlled using an acousto-optical tunable filter (AOTF, Gooch & Housego). Oblique illumination (near total internal reflection) was employed to enable deep 3D imaging while maintaining a relatively low background. For 3D imaging, a cylindrical lens with a focal length of 1000 mm was introduced into the detection path to create astigmatism for axial localizations. Calibration data were collected before 3D imaging using 100-nm-diameter fluorescent beads (Molecular Probes) while scanning in the z direction. Samples were imaged in a freshly prepared switching buffer containing 10% glucose, 100 mM tris pH 8, 1% BME and 1% GLOX (glucose oxidase/catalase). Image stacks (also termed as “movies”) were captured using an electron-multiplying charge-coupled device camera (iXon+DU897, Andor) via custom acquisition software at 200 Hz for 50,000-400,000 frames with an illumination power density of ~ 3.5 kW/cm². In case of multichannel imaging, an initial reference image of all structures simultaneously labeled with the same dye using their corresponding adapter and reporter DNA oligonucleotides were obtained for registration among channels. A 405 nm activation laser (OBIS LX, Coherent) was used to enhance the photoactivation rate of Alexa Fluor 647. An aspirator was used to perform on-stage liquid exchanges (see Supplementary Figure 3.11).

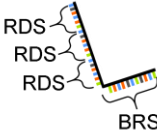
3.8.8 *Data analysis*

STORM images were generated using similar methods as previously described⁴⁹. Briefly, for 2D STORM, images of individual molecules were identified and fitted to a 2D Gaussian function to obtain the centroid coordinates (x_0, y_0), which were assigned the positions of the single molecules for reconstructing a STORM image. For 3D STORM, an elliptical Gaussian function was used instead. The lateral coordinates of molecules were determined in the same way using the Gaussian centroid coordinates, and the vertical coordinate (z) was calculated by comparing the Gaussian widths (w_x, w_y) to the calibration curves. Focal shift and spherical aberration corrections were carried out at each focal plane as described previously⁵¹. Lateral and axial drift correction was then applied to the super-resolution image based on cross-correlations of adjacent subsets of localizations. Examination of the cross-correlation trajectories used to correct for drift in data analysis revealed an axial drift of ~50-100 nm and a lateral drift of ~250-500 nm during STORM movie acquisition whether or not refresh steps had been previously performed. This indicated that the brief rinse with hot water during the melting step for probe refresh (which was followed by much longer washes and hybridization steps at room temperature) did not lead to serious drift during the subsequent imaging step. For multilayer 3D images, localizations ~300 nm beneath the focal plane were accepted, while localizations above the focal plane were rejected due to their poorer spatial resolution resulting from spherical aberration correction. Prior to merging all layers together, alignments in three dimensions were carried out using a Fourier transform based cross-correlation method. To register images laterally, all images to be aligned were saved as high resolution x-y projections. Then the 2D cross-correlation between two adjacent layers was calculated and images were then aligned by translating all localizations by the displacement between the position of the cross-correlation peak and the origin. After lateral alignment, identical x-z slices of each movie were saved and x-z alignment was carried out using the same cross-correlation procedure. In some cases, lateral and axial registration processes were iterated to ensure good alignment in all three dimensions. In the case of multichannel registration, an image of all structures served as a reference image for alignment among channels. Here, all targets to be imaged were simultaneously labeled with the same dye using their corresponding adapter and reporter DNA sequences. Images in each channel in the same layer were aligned to

the reference image using the same Fourier transform cross-correlation method, followed by multilayer alignment in each channel as in the case of single channel multilayer 3D imaging.

3.9 SUPPLEMENTARY FIGURES AND TABLES

Supplementary Table 3.1. DNA Sequences.

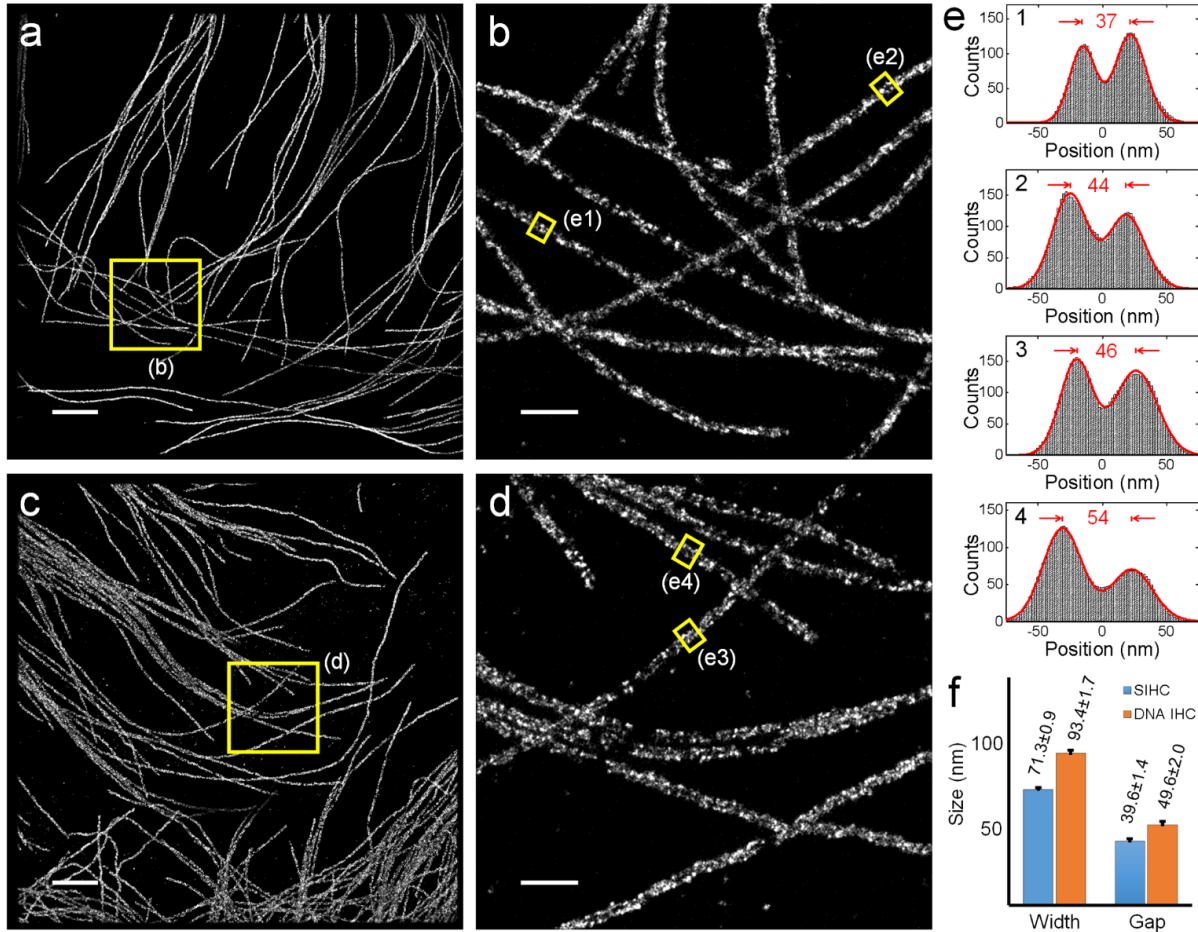
Secondary Antibody and Sequence 5'-3'	Adapter Sequence 5'-3'	Reporter Sequence 5'-3'
		
Donkey anti Rat min Mouse / 5AmMC6 / GCATTACAGTCCTCATAAGT (LAG_010)	ACTTATGAGGACTGTAATGCTTA ATGATACGGCGACCACCGATTAA TGATACGGCGACCACCGATTAA GATACGGCGACCACCGA (LAG_013)	/5A1ex647N/TCGGTGGT CGCCGTATCATT (P5A647JV)
Donkey anti Rabbit / 5AmMC6 / CCGAATACAAAGCATCAACG (LAG_007)	CGTTGATGCTTTGTATTCGGTTA ATGATACGGCGACCACCGATTAA TGATACGGCGACCACCGATTAA GATACGGCGACCACCGA (LAG_015)	/5A1ex647N/TCGGTGGT CGCCGTATCATT (P5A647JV)
Donkey anti Mouse min Rat / 5AmMC6 / GACTACTGATAACTGGATTG (LAG_017)	CAATCCAGTTATCAGTAGTCTTA ATGATACGGCGACCACCGATTAA TGATACGGCGACCACCGATTAA GATACGGCGACCACCGA (LAG_016)	/5A1ex647N/TCGGTGGT CGCCGTATCATT (P5A647JV)

Notes: Colors of the sequences in adjacent columns indicate complementarity. The code in parentheses below each sequence indicates the sequence name. Each adapter contains space for three reporter strands to bind, and all adapters bind to the same reporter strand. BRS: barcode recognition sequence; RDS: reporter docking sequence; RS: reporter stand; Dye: Alexa Fluor 647.

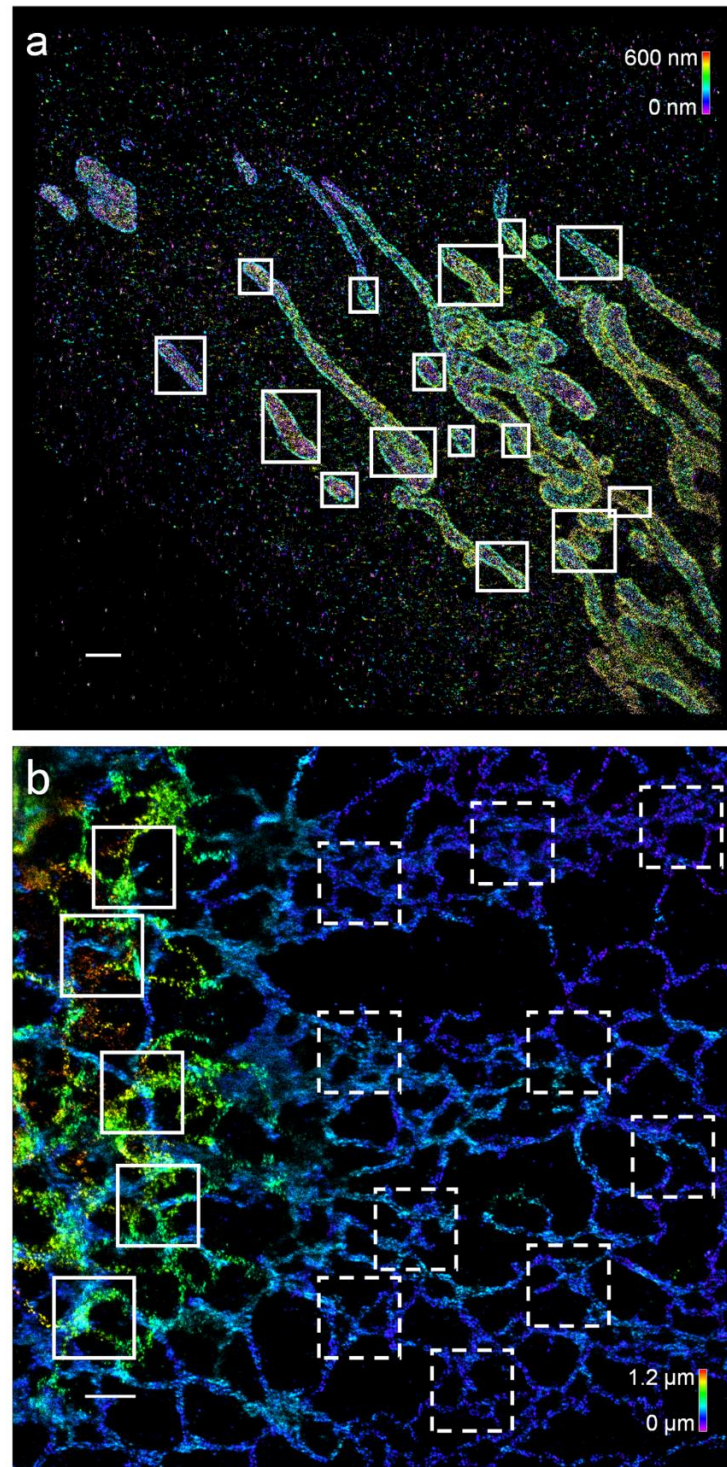
Supplementary Table 3.2. Summary of sample preparation and imaging conditions.

Fig.	Specimen	Extraction	Primary Ab (1-2 µg/mL)	Secondary Ab (8-12 µg/mL)	Adapter(s)	Rounds ¹	Thickness	Total Time ⁴	Total Localizations	Imaging Conditions
1	COS-7 cell, wildtype	saponin	Rb x TOM20	D x Rb-LAG_007	LAG_015	13	3.2 µm	4.8 hr	274,613	200 Hz, 100k frames/round, 100x 1.45 NA oil objective, 405 activation
2a-b	BS-C-1 cell, wildtype	none	Rb x TOM20	D x Rb-LAG_007	LAG_015	10 (b)	600 nm	42 min (no refresh) 3 hr (refresh)	14,237 (no refresh) 51,350 (refresh)	200 Hz, 50k frames/round, 100x 1.45 NA oil objective
2d	BS-C-1 cell expressing Sec61β-GFP	none	Rb x GFP	D x Rb-LAG_007	LAG_015	4	1.2 µm	33 min (no refresh) 3 hr (refresh)	886,678 (no refresh bottom to top) 414,528 (no refresh top to bottom) 4,982,997 (refresh)	200 Hz, exhaustion ⁵ , 100x 1.45 NA oil objective, 405 activation
3	COS-7 cell expressing Sec61β-GFP	saponin	Ms x GFP	D x Ms-LAG_017	LAG_016	9	1.5 µm	4.5 hr	15,753,747	200 Hz, 200k frames/round, 100x 1.45 NA oil objective 405 activation
4	COS-7 cell expressing Sec61β-GFP	saponin	Rat x Tub Rb x TOM20 Ms x GFP	D x Rat-LAG_010 D x Rb-LAG_007 D x Ms-LAG_017	LAG_013 LAG_015 LAG_016	4x3 ²	800 nm	6.3 hr	5,725,543 2,128,887 2,518,580	200 Hz, 200k frames/round, 100x 1.45 NA oil objective 405 activation
S1	BS-C-1 cell, wildtype	triton	Rat x Tub	D x Rat AF647 D x Rat-LAG_010	N/A LAG_013	1	~600 nm (2D only)	17 min	2,093,518 (a) 1,667,061 (c)	200 Hz, 200k frames/round, 100x 1.45 NA oil objective 405 activation
S2a	BS-C-1 cell, wildtype	none	Rb x TOM20	D x Rb-LAG_007	LAG_015	10	600 nm	42 min (no refresh) 3 hr (refresh)	980,303	200 Hz, 50k frames/round, 100x 1.45 NA oil objective
S2b	BS-C-1 cell expressing Sec61β-GFP	none	Rb x GFP	D x Rb-LAG_007	LAG_015	4	1.2 µm	33 min (no refresh) 3 hr (refresh)	4,982,997	200 Hz, exhaustion ⁴ , 100x 1.45 NA oil objective, 405 activation
S3	BS-C-1 cell, wildtype	triton	Rat x Tub	D x Rat AF647 D x Rat-LAG_010	N/A LAG_013	20	~600 nm (2D only)	5.9 hr	384,956 (a) 439,084 (b)	500 Hz, 100k frames/round, 60x 1.2NA water objective 405 activation
S4	COS-7 cell, wildtype	saponin	Rb x TOM20	D x Rb-LAG_007	LAG_015	13	3.2 µm	4.8 hr	1,816,838	200 Hz, 100k frames/round, 100x 1.45 NA oil objective, 405 activation
S6	COS-7 cell, wildtype	saponin	Rat x Tub Rb x TOM20 Ms x αTub	D x Rat-LAG_010 D x Rb-LAG_007 D x Ms-LAG_017	LAG_013 LAG_015 LAG_016 Reporter only	1x12 ³	~600 nm (2D only)	3 hr	518,014 (tubulin) 363,875 (mitochondria) 314,149 (ER)	200 Hz, 50k frames/round, 100x 1.45 oil objective

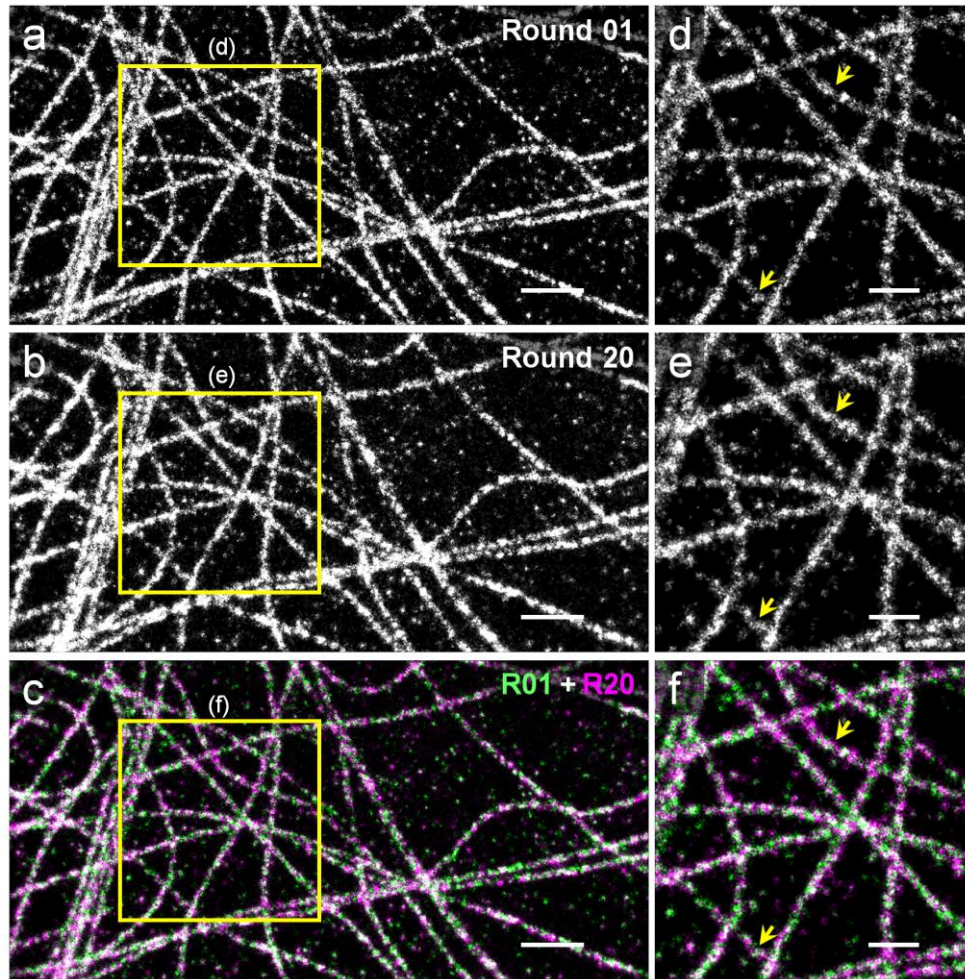
Notes: ¹Number of rounds, where each round consists of melting, hybridization, and washing steps (Figure 3.1), but where the first round omits the melting step. ²In Figure 3.4, “4x3” means 4 rounds for 3 channels. ³In Supplementary Figure 3.10, “1x12” means 1 layer for 12 cases. ⁴Total times were estimated based on a conservative 15 minute melt/hybridization period per round of imaging along with imaging time determined from the number of frames and frame rate. ⁵“Exhaustion” here means imaging until nearly all the dyes were photobleached, typically at ~400,000 frames when gradual 405 nm laser activation was used during imaging.



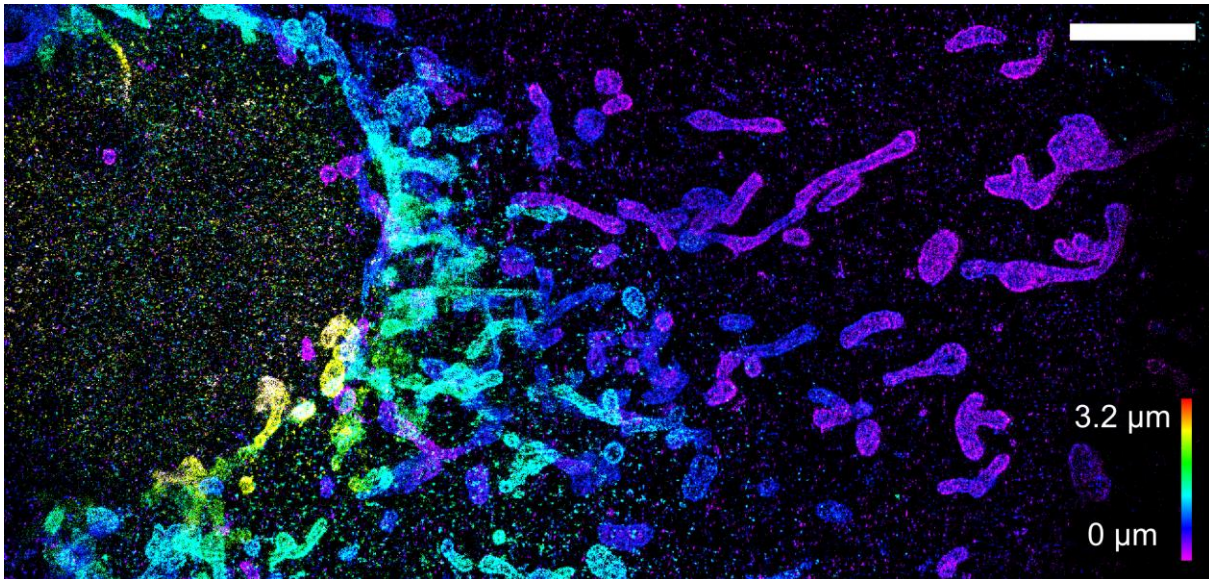
Supplementary Figure 3.5. Comparison of tubulin immunostains in BS-C-1 cells using either standard fluorescent secondary antibody or DNA-conjugated secondary antibody. **a)** 2D STORM image using a standard fluorescent secondary antibody. **b)** Zoomed-in view of box in **a**. **c)** 2D STORM using a DNA-conjugated secondary antibody. **d)** Zoomed in view of box in **c**. **e)** Examples of histograms of number of localizations in cross-sections of microtubules in both cases for boxed regions in panels **b** and **d**. **f)** Statistical results on 59 cross-sections in **a** and 58 cross-sections in **c**. Data were expressed as mean $\pm$ standard error of the mean. Contrast has been adjusted individually in **a-d** for better visualization of the tubule structures; the number of localizations per unit length of microtubules was $\sim$ 30% larger for standard fluorescent secondary antibodies compared to DNA-conjugated secondary antibodies. Scale bars: 2 μ m (**a**, **c**), 500 nm (**b**, **d**).



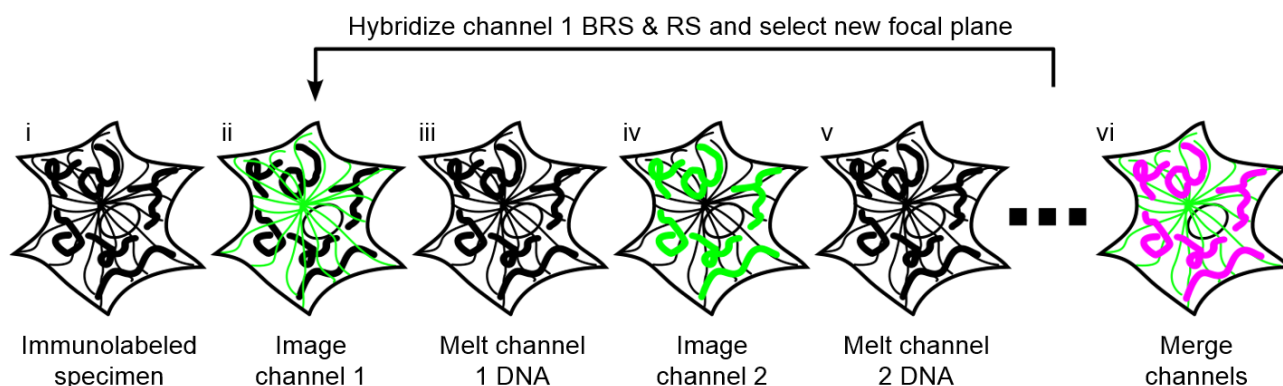
Supplementary Figure 3.6. Regions of interest selected for statistical analysis shown in Figure 3.2. **a)** Positions and sizes of the 15 random regions for analysis in Figure 3.2c, i.e., in single-layer 3D STORM images of mitochondria with and without probe refresh steps. **b)** Positions and sizes of the 5 thick regions (solid boxes) and 10 thin regions (dashed boxes) for analysis in Figure 3.2e, i.e., in 4-layer 3D STORM images with and without probe refresh steps. Scale bars: 1 μm.



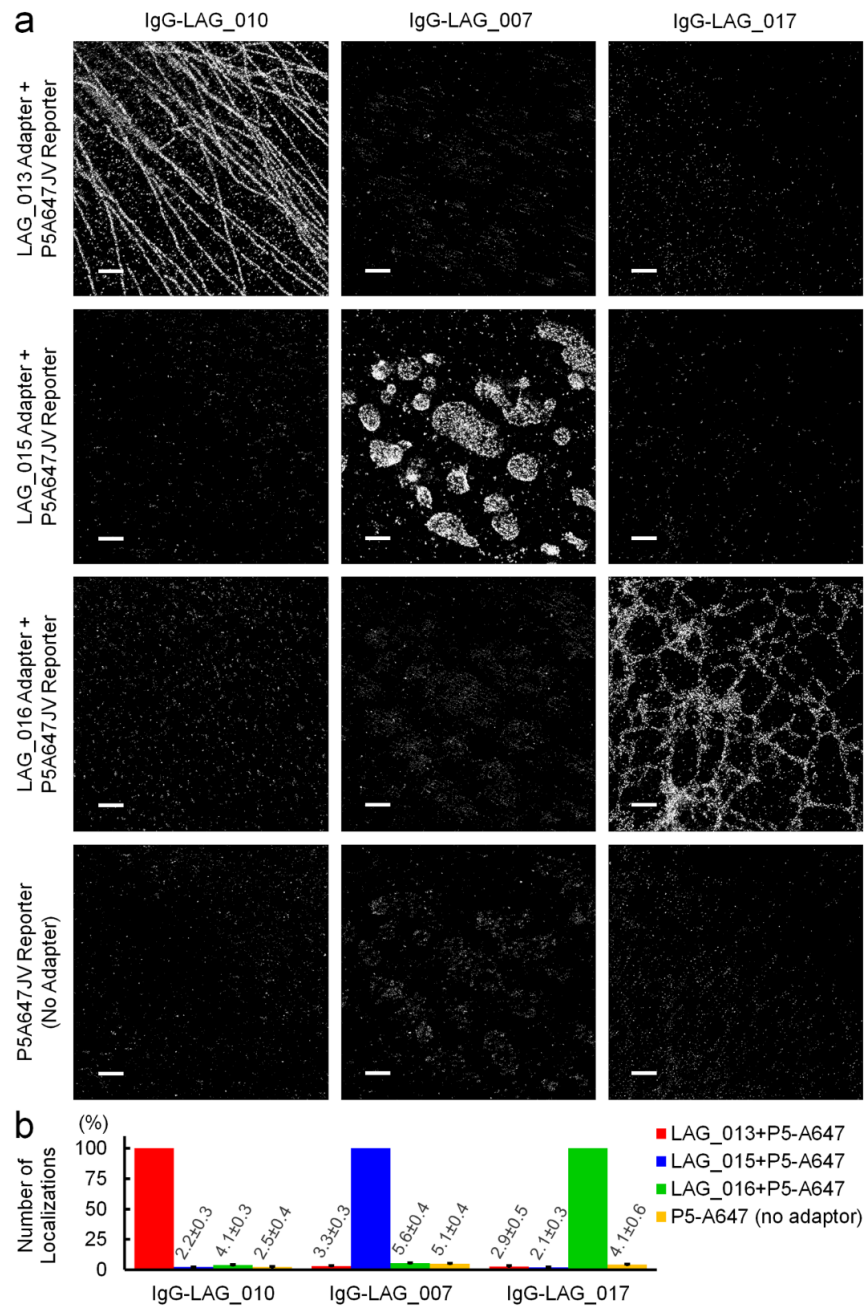
Supplementary Figure 3.7. Assessment of structural preservation in prSTORM. **a)** 2D STORM image (Round 01) of microtubules in a BS-C-1 cell immunostained for α -tubulin. **b)** 2D STORM image (Round 20) of the same region after repeating 19 additional rounds of melting, hybridization, and washing. **c)** Overlay of panels **a** and **b**. **d-f)** Zoomed-in views of boxes in **a-c**. Scale bars: 1 μ m (**a-c**), 500 nm (**d-f**). Arrows indicate two regions showing minor distortions after 20 rounds of melting, hybridization, and washing.



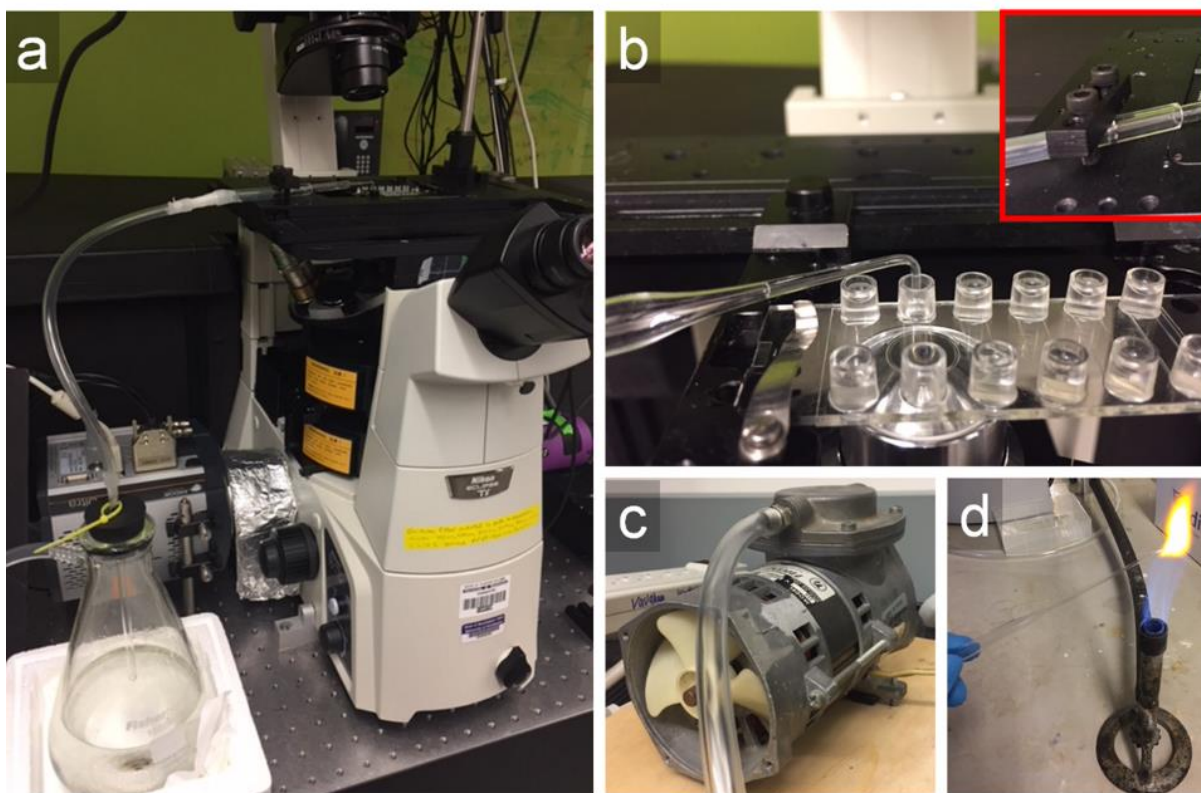
Supplementary Figure 3.8. Mitochondrial network in a COS-7 cell at a depth of 3.2 microns, with 13 layers and 100K frames per layer using prSTORM (a small region of this data set was also shown in Figure 3.1). Imaging with conventional immunohistochemistry is limited to ~400K frames, but with prSTORM the fluorescence was refreshed permitting the structure to be densely sampled over an extended depth. Scale bar: 4 μm .



Supplementary Figure 3.9. prSTORM schematic adapted for imaging of multiple channels. i) A specimen is immunolabeled with 2 (or more) sets of antibodies containing unique DNA barcodes. ii) BRS and RS corresponding to the DNA barcode for channel 1 is hybridized to the sample so that one of the structures is labeled. Channel 1 (microtubules, green) is imaged at a desired focal plane. iii) The BRS and RS for channel 1 is melted off using hot water. iv) BRS and RS corresponding to channel 2 is hybridized to the sample and an image is taken at the same focal plane as channel 1. v) The BRS and RS for channel 2 is melted off using hot water. This sequence can be repeated to image all channels at the same focal plane as channel 1, after which BRS and RS corresponding to channel 1 is hybridized on the sample and a new focal plane is selected. Steps ii-v are repeated until all channels have been imaged at all focal planes of interest in the specimen. vi) All channels are aligned in x, y, and z then merged into one image.



Supplementary Figure 3.10. Cross-talk analysis for secondary antibodies for prSTORM used in this study. **a)** 2D STORM images, with the left, middle, and right columns showing cells immunostained for α -tubulin, TOM20, and sec61 β -GFP, respectively. In successive rows, the specimen has been washed with hot water to remove adapter/reporter oligonucleotides, incubated with the indicated adapter/reporter oligonucleotides, washed, and imaged. Within each column, the same region of each cell is imaged. Scale bars: 1 μ m. **b)** Quantitative analysis. The number of localizations was determined for each condition and normalized to the positive (bright) channel in each column. Because this approach counts localizations resulting from nonspecific binding, the crosstalk values here likely represent an upper bound to the crosstalk. Data were expressed as mean $\pm$ standard error of the mean.



Supplementary Figure 3.11. Simple stage-top flow channel apparatus for probe refresh STORM. **a)** An aspirator flask is connected to a vacuum pump and a Pasteur pipette that is secured above a flow channel containing fixed and immunostained cells. **b)** Close-up of Pasteur pipette with bent tip. The Pasteur pipette is positioned so that its tip is ~2-3 mm above the bottom of the flow channel without touching any part of the well. The inset shows how the Pasteur pipette is secured to the movable portion of the microscope stage so that it moves with the specimen. The unoccupied well across from the Pasteur pipette is used to deliver liquids into the channel by hand pipetting. When the vacuum pump **c)** is powered on, excess liquid from the flow channel is removed without sucking air bubbles through the channel. The vacuum pump is turned off during liquid incubations and data acquisition. **d)** Heating the tip of a Pasteur pipet over the flame of a Bunsen burner to bend its tip.

CHAPTER 4. STRUCTURALLY-DENSE IMAGING WITH ULTRA-LOW DUTY CYCLES USING PRSTORM

4.1 PREFACE

This next chapter is incomplete and addresses the goals and current progress in using prSTORM to image dense structural regions. While the concept of what we are trying to achieve is somewhat simple, there have been several technical challenges that we are still in the process of resolving. To execute the experiments I have had to redesign and rebuild our microscope's focus lock mechanism along with an on-stage fluidics system to mitigate the potential risks of hand-pipetting many times during data acquisition. In doing so this called for new data acquisition software to integrate the fluidic components of the experiment with data acquisition.

We also had to rethink our sample preparation in terms of alignment of consecutive rounds of imaging with data sets that have a sparse number of localizations. In our previous work detailed in **Chapter 3** we used cross-correlation between adjacent data sets in order to align movies. In the case of our new experiments, however, we have not been able to use cross-correlation for alignment due to the much sparser nature of our data. To this end we have explored a variety of fiduciary markers and procedures for image alignment, many of which exhibited inconsistent drift within the sample due to the washing steps in our experiments. We finally found commercially-available coverslips containing gold nanoparticles (Hestzig LLC) which seem to provide uniform luminescence signals suitable for image alignment. Separately, another hurdle has been establishing an efficient data analysis pipeline for aligning many movies. The data analysis pipeline has largely been the focus of my research partner Marco Howard while I have been focused on instrumentation, sample preparation, and data collection. We are currently optimizing the experimental conditions (e.g., number of imaging rounds, frames per round, labeling concentrations, etc.) and the data analysis pipeline.

4.2 INTRODUCTION

While single molecule localization microscopy (SMLM) has provided a revolutionary tool to researchers worldwide since its creation in 2006^{19,20,22,33}, there are still important aspects of the technique that need improvement in order to reach a wider user base. One serious drawback of SMLM that places a limitation of its user base has been the reliance on a fluorophore's photoswitching properties to generate single-molecule localizations. The properties, like duty cycle and number of detected photons per localization, not only limit the spatial resolution achievable by SMLM but also limit regions that can be imaged effectively with SMLM. I will illustrate this point with microtubules as an example however the principles apply to all structures.

A key indicator of a high-quality microtubule image is hollowness running throughout at least some portions of the structure with a separation of peaks of ~ 40 nm. This hollowness arises from properly localizing labels around the circumference of the microtubule (Figure 4.1). Since the antibody-labeled microtubule structure is that of a hollow tube or cylinder, when projected

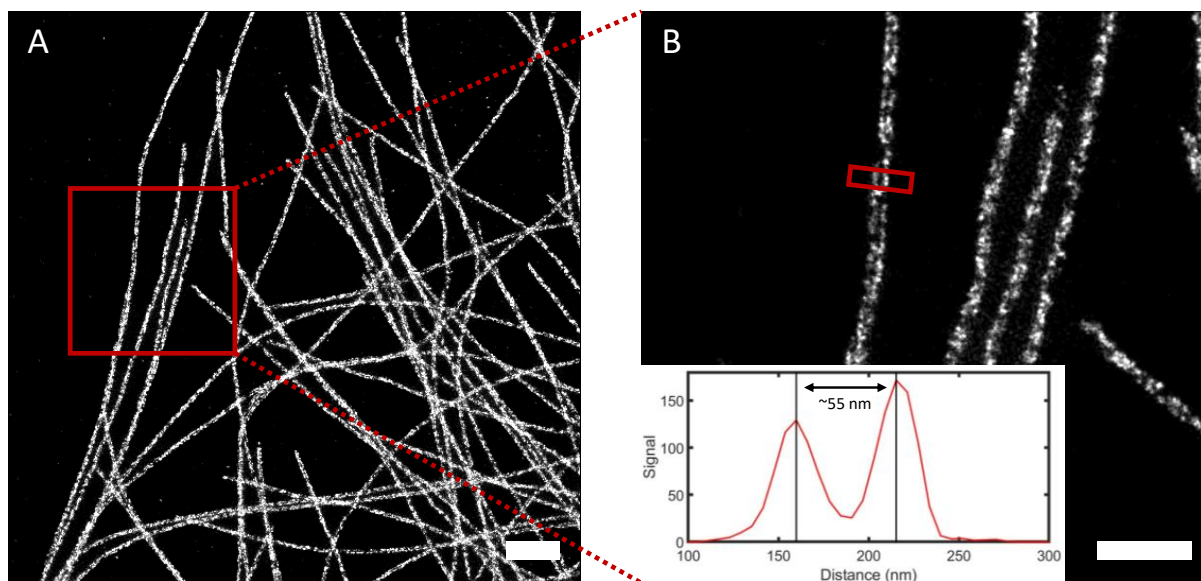


Figure 4.1. Hollowness of Microtubules. A) Zoomed-out view of microtubules in fixed COS-7 cells labeled with secondary antibodies bearing Alexa 647 fluorophores. B) Zoom-in of boxed region in A, showing hollow features of the microtubules. The inset corresponds to the signal measured across the boxed region of B and the peak-to-peak distance represents the measured microtubule width in this section. The distance is slightly broadened due to the addition of DNA on the secondary antibodies. Scale bars: 1 μ m (A); 500 nm (B).

in two dimensions, it produces a hollow feature^{30,31,112,119} as long as fluorescently labeled and densely distributed secondary antibodies are properly localized on the surface. A typical high-quality super-resolution image of microtubules will usually consist of a relatively flat region of the cell with a patch of fairly sparse microtubules. There will be clear intersections where, even at the conventional level, it is not too difficult to ascertain the number of microtubules crossing. By selecting a region like this, a properly prepared and imaged specimen should produce smooth and hollow microtubules.

The density of microtubules is an important property to consider when selecting a region to image. If the region is too dense, meaning there are a lot of overlapping and crossing microtubules, the image quality can suffer. This is due to the limitations imposed by the fluorophore's duty cycle, the fraction of time a fluorophore spends in the on-state. The duty cycle relates directly to the density of objects that can be imaged with SMLM.

Consider a diffraction-limited region of low structural density containing one microtubule, and a diffraction-limited region of high structural density containing five microtubules. In order to accurately resolve the structures, during any given imaging frame there needs to be one fluorophore or less in the on-state within this diffraction limited region. When there are a lot of densely labeled structures in a single diffraction limited region the probability of multiple fluorophores being in the on-state at any one time increases substantially. Even with the best-performing organic fluorophore for SMLM, Alexa 647, this is the case. This is demonstrated by the multiple localization likelihood (MLL) which quantifies the probability of more than one fluorophore in a diffraction-limited region being on at the same time. The equation for MLL is, $MLL = 1 - (1 - DC)^{N-1}$, where DC represents the fluorophore's duty cycle and N represents the number of fluorophores in a diffraction-limited region. In this scenario, the region of low structural density with one microtubule would have an MLL of 0.006 if imaged with Alexa 647 (DC = 0.001) and there 60 fluorophores in the region. In comparison, the structurally-dense region that contains five times more fluorophores ($N = 300$) would have an MLL of 0.26 or a 26% chance of having multiple localizations. A possible alternative to this regime is to lower the number of labels on the sample to decrease the probability of multiple diffraction limited fluorophores being in the on-state at the same time. However, this may place a limit on resolution

by not being able to fulfill the Nyquist criterion which relates the highest possible resolution to the average distance between two adjacent labels⁶⁶.

When multiple fluorophores exist in the on-state within a diffraction limited region at the same time it gives rise to false localizations and localization artifacts^{176–178}. While Alexa 647 is a workhorse for SMLM, even at a structurally sparse level, the limitations imposed by duty cycle are noticeable. When observing two microtubules crossing it would be expected that at the intersection there would be twice the number of localizations than in a region of the same size along a single microtubule. This is true when imaging using conventional widefield fluorescence, there is twice as much signal at the intersections than along a single microtubule. However, when imaging with SMLM the localization numbers do not reflect this observation (Figure 4.2). This phenomenon can be explained by an increase in multiple localization errors at the intersections which are (mostly) discarded during image analysis. A multiple localization error arises from two or more fluorophores being detected within a diffraction-limited volume. Some of these produce an image with a width that is larger than the instrument's point spread function (PSF). This means that multiple fluorophores in a diffraction limited region were in the on-state at the same time

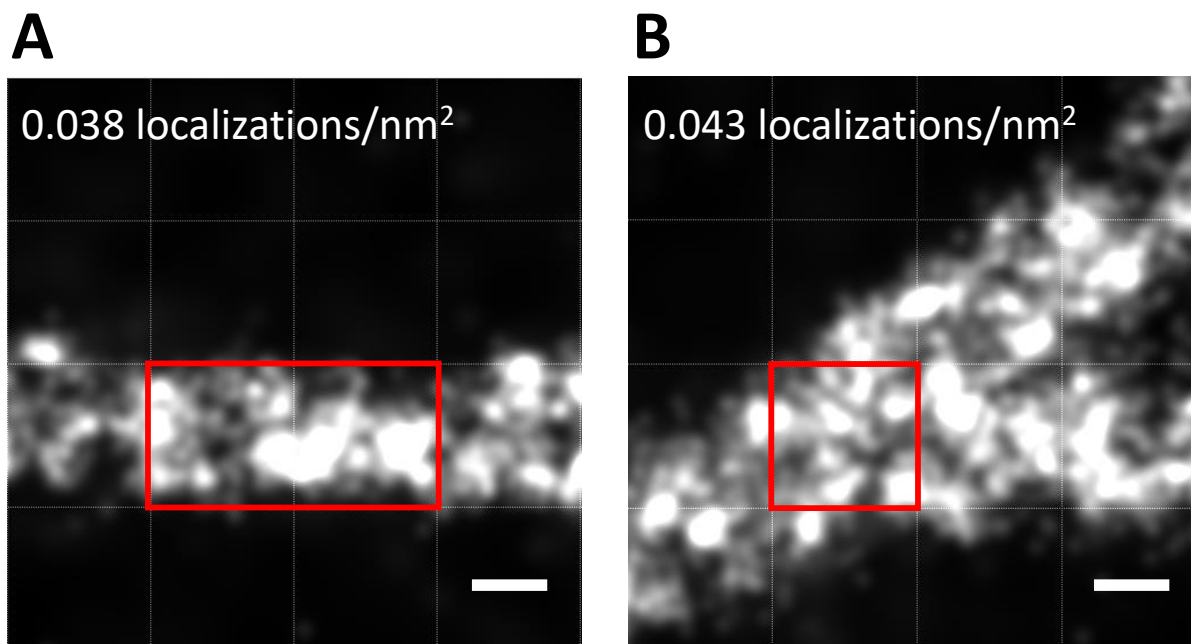


Figure 4.2. Localization density comparison. A) Average localization density of single molecules in the boxed region containing a single microtubule. B) Average localization density of the boxed region where two microtubules intersect. Scale bars: 50 nm.

resulting in a larger spot size. The center position becomes the average of the contributing fluorophores rather than the actual position of any of the individual fluorophores. This effect becomes even more pronounced in regions of high structural density and imposes inherent limitations on what types of structures and regions can be imaged with SMLM. Structures with diffraction-limited features that are relatively sparse throughout a sample are ideal, however, researchers needing to resolve features in structurally dense regions of a sample will have difficulty obtaining desirable results with current implementations.

In the remainder of this Chapter I will present the progress of my current work which utilizes prSTORM (described in **Chapter 3**) for artificially lowering the duty cycle of Alexa 647 in order to enable imaging of dense structural regions with minimal localization artifacts. This method could also be applied to other fluorophores in order to improve their duty cycles but is not to focus of this work.

4.3 ULTRA-LOW DUTY CYCLE SMLM

I propose that by combining fluorophore blinking properties with DNA binding properties I will be able to achieve ultra-low duty cycles with SMLM. In contrast to other methods using DNA binding, such as DNA-PAINT, our DNA probes stably bind to the specimen throughout the imaging duration and the localizations are generated by the photoswitching properties of the fluorescent labels. Under normal conditions we aim to try and label all the possible DNA binding sites with DNA bearing a fluorescent tag by exposing the specimen to high concentrations of reporter DNA and allowing sufficient time for the DNA binding to reach equilibrium. Here, the idea is to collect many rounds of a small number of frames containing a sparse subset of possible localizations. The sparse subset of localizations is achieved by adjusting the reporter DNA concentration and/or hybridization such that only a small number of possible DNA binding sites are occupied by DNA bearing the fluorophore (Figure 4.3). Once many rounds of imaging are obtained the individual movies are combined to generate one image with reduced multiple localization errors. In the context of the MLL equation, the value of N is being broken up into small fractions thereby reducing the overall MLL without actually reducing the number of labels on the specimen. This has the effect of lowering the number of labels while still fulfilling the Nyquist criterion.

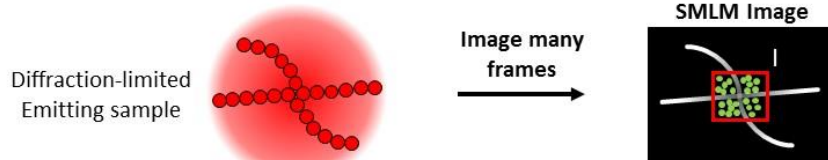
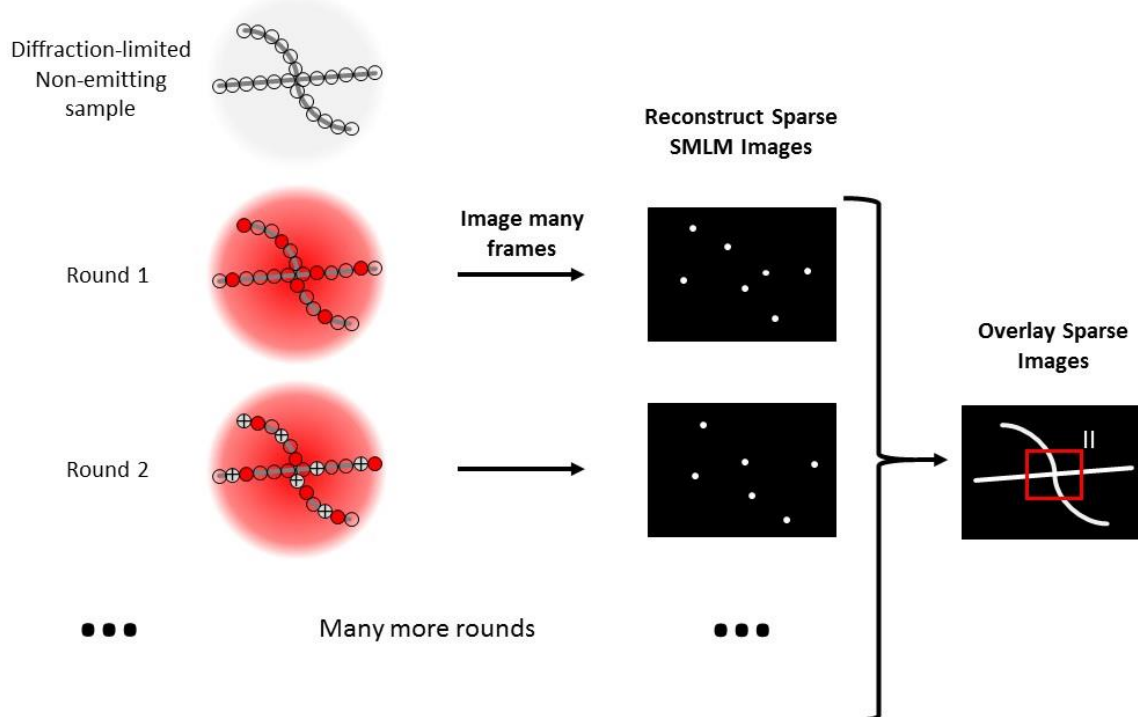
A) Standard SMLM**B) Ultra-low duty cycle SMLM**

Figure 4.3. Schematic for achieving ultra-low duty cycles with prSTORM. A) A fluorescently-labeled diffraction-limited sample represented by the two crossing filaments surrounded by a hazy red circle is imaged using standard SMLM. The resulting super-resolution image is on the right. B) The same diffraction limited sample is labeled densely but not emitting any fluorescence. In round 1 a sparse subset of labeled sites are capable of emitting fluorescence, represented by a few circles being filled in with red. These probes are imaged using SMLM and a sparse SMLM image is reconstructed. In round 2 the previous round's labels have photobleached, represented by grey circles with crosses in them. A new small population of remaining labels are now capable of generating fluorescent signal and are imaged. This process is repeated many times and then the sparse SMLM images are overlaid to generate a single SMLM image. I) The boxed region highlights the crossing of the two filaments, here there is a loss in signal along the filaments and presence of many false localizations represented by green dots, and both are a result of multiple localization errors. II) The boxed region shows uniform signal throughout and no false localizations.

There are a few potential ways to achieve the sparse labeling for this method. In our currently preferred method to artificially lower the duty cycle of Alexa 647, secondary antibodies are first labeled with single-stranded DNA bearing a unique DNA barcode as previously reported¹⁷⁹. Adapter DNA containing three potential docking sites for reporter DNA strands is hybridized on the specimen for 10 minutes at a concentration of 200 nM and then rinsed with PBS. Reporter DNA bearing Alexa 647 is then exposed to the sample for 60 seconds at a concentration of 40 nM and immediately rinsed off with PBS. This results in only a sparse subset of the possible reporter DNA binding sites being occupied at a given time. The specimen is then imaged with a reduced probability of adjacent fluorophores being on in a given diffraction limited region at one time. After imaging, the fluorophores have photo-bleached and more reporter DNA is exposed to the sample so a new subset of fluorophores can be imaged. The process is repeated many times until a sufficient number of localizations have been obtained to construct a super resolution image. If a noticeable drop in localization numbers occurs it is an indication that the binding sites are getting filled up and the sample should be refreshed. This can be done by simply flowing hot water on the specimen to denature the hybridized DNA strands and then rinsing with PBS followed by a 10 minute incubation of fresh adapter DNA. This approach is convenient in that it allows for short hybridization times and thus allows for faster overall data acquisition.

The alternative approach to achieve sparse localizations involves imaging at equilibrium which means hybridizations will require a waiting period of around 10 minutes. In this regime there is a sample labeled with DNA labeled antibodies, the same as in the previous scenario. Next, solution containing pre-hybridized reporter and adapter DNA is exposed to the sample for 10 minutes at which point equilibrium has been established. This is identical to the protocol for prSTORM as described in **Chapter 3**, however, in this case, the reporter DNA consists of a ratio of fluorophore-labeled DNA and unlabeled DNA. The sample is then imaged after which hot water is used to remove all DNA from the specimen and the process is repeated many times.

4.4 AUTOMATED FLUIDICS FOR EFFICIENT ON-STAGE LIQUID HANDLING

To minimize human error and increase imaging efficiency, I constructed an automated fluidics system to eliminate the need for pipetting of liquids while the sample is on the stage. This eliminates the risk of bumping the sample halfway through imaging, which would ruin the experiment. It also allows the researcher the ability to step away from the instrument while data is still being collected without interrupting the experiment.

The fluidics system is based partly on a previously published design¹⁸⁰ with several modifications to better suit this application (Figure 4.4). In brief, liquids are fed into an 8-way valve which is connected to the sample that sits on the microscope stage. The sample is then connected to tubing which passes through a peristaltic pump going to the waste container. Both the pump and valve have been integrated into LabVIEW data acquisition software to allow for sequences of operations to be executed that control valve positioning and pump speed, along with data acquisition processes.

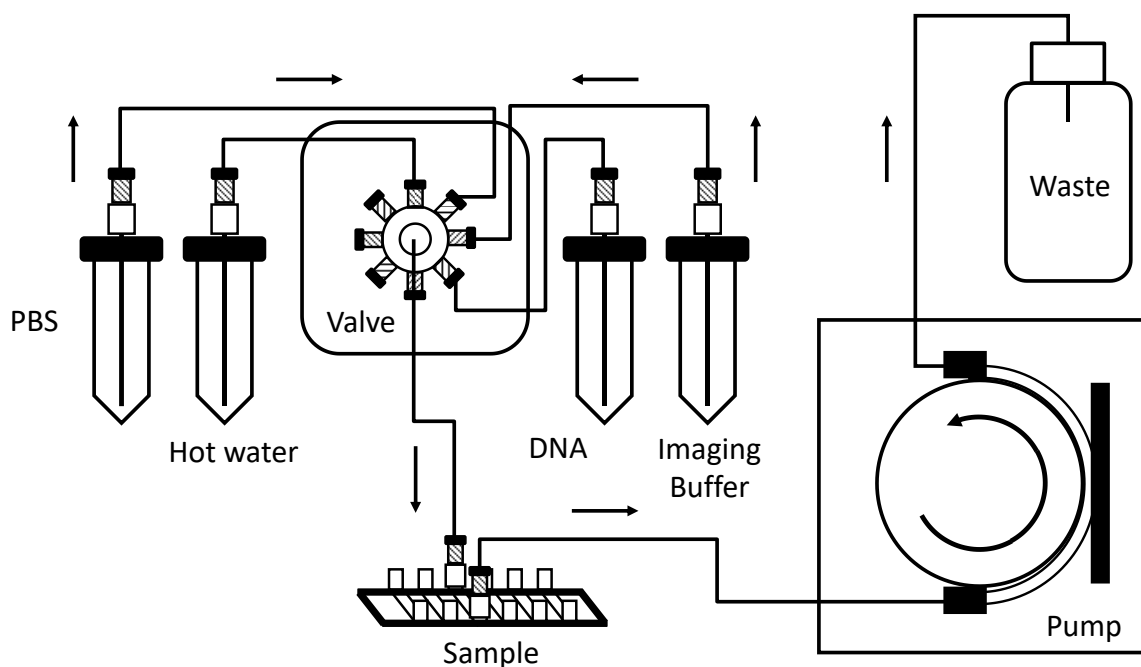


Figure 4.4. Automated fluidics. Solutions containing PBS, hot water, DNA, and imaging buffer are connected to an eight-way valve going to the sample. Liquid is pulled through the specimen using a peristaltic pump located after the sample which pushes liquid to the waste container.

Modifications to the previously published design¹⁸⁰ of this setup are primarily around the sample (Figure 4.5). The previous implementation used an open chamber design, while, here, I used ibidi flow channels (along with the necessary tubing components) in order to minimize sample volumes. In addition, shut off valves were placed before and after the sample to enable easy removal and swapping of samples with minimal leaking of tubes and development of air bubbles. Efforts were also made to reduce strain on the sample and prevent components from interfering with the microscope's ability to maintain focus. A precautionary objective lens "raincoat" was made by cutting a hole in the middle of a latex dental dam and securing it around the objective. In the event of any leaks this would prevent liquid from damaging the expensive optics and piezoelectric Nano positioner. A consumer-grade hot water boiler capable of maintaining water at 85°C was adapted to fit the tubing by drilling a hole through the plastic lid

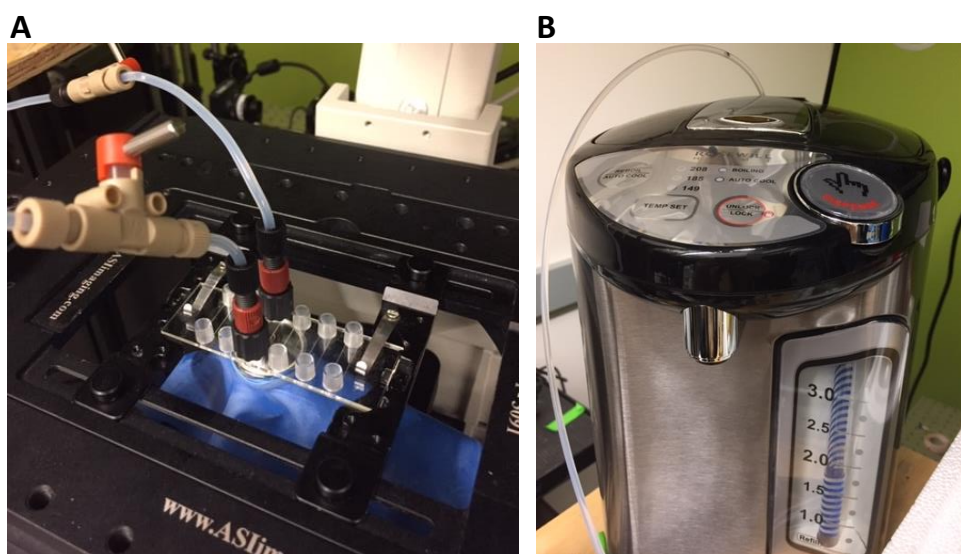


Figure 4.5. Modifications to original design. A) Input and output tubing connected to an ibidi flow channel imaging chamber sitting on the microscope stage. Shut off valves on either side of the chamber provide a simple way to swap samples on and off the stage without leaking from tubes. A jacket (blue) is placed around the objective and over the piezo Nano positioner to prevent damage in case of any leaks. B) A hot water boiler modified to supply hot water to the system.

and feeding the heat resistant tubing through its center. The design was implemented in a top down approach where the liquids and valve were positioned on a table elevated slightly above the microscope stage and the pump/waste components were positioned at a level lower than the stage. This reduces the potential for air bubbles forming and reaching the sample, a situation

which could result in damage to the sample or disruption of an experiment if a pocket of air gets stuck in the flow channel.

4.5 COMPUTER-PERISTALTIC PUMP INTERFACE

It is worth noting that making a physical connection between the peristaltic pump (Gilson Minipulse 3) and computer is not straightforward. The connections offered on the pump are proprietary to the manufacturer. The pump can be connected to a computer by purchasing a somewhat expensive “black box” component which converts the pump output to RS 232 which can be connected to the computer. A cheaper alternative to the black box is to connect the pump GSIOC (Gilson Serial Input/Output Channel) output to a RS485/422 to RS 232 converter and then connect that to a RS 232 to USB converter which can connect to the computer (Figure 4.6)

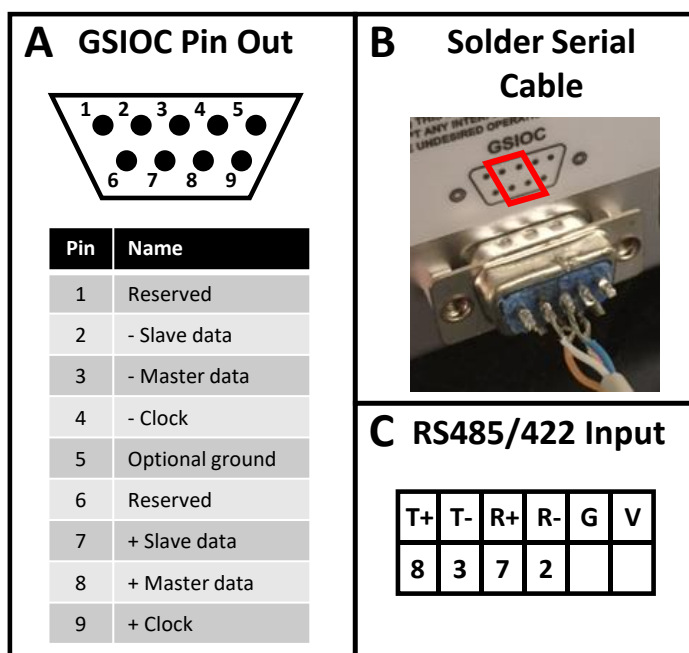


Figure 4.6. Making GSIOC compatible with RS232. A) The pinout and manufacturer designations for the GSIOC male connection. B) Solder a serial female to male adapter to have wires connected to pins 2, 3, 7 and 8 (boxed in red). C) Connect the wires coming from the designated pins to the appropriate RS485/422 to RS 232 converter input slot as shown.

This concludes the current progress of this work which will be handed off to Marco Howard after my departure from the program.

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APPENDIX A

Included here is documentation pertaining to macro operations in LabVIEW programming used for data acquisition and fluidics control in Chapter 4.

Command	Definition	Notes	P(0)	P(1)	P(2)	P(3)	P(4)	P(5)	P(6)
Init	Start custom sequence mode	Use at start of every sequence							
Exit	End custom sequence mode	Use at end of every sequence							
Load	Loads a configuration file		Config to load ¹	Load into destination 0 = CCD 1 = Laser 2 = Filter wheel 3 = Motion					
Move	Moves the stage	Requires all 4 parameters	Selects stage to be moved	Desired X position	Desired Y position	Desired Z position (absolute position)			
Wait	Wait period before going to next step		Time in seconds						
Goto	Go to step and loop		Step # to jump to	Current iteration in loop ²	Desired number of iterations				
Setpar	Sets a parameter to a particular value	Use to initialize Goto P(1) value to 0	Step #	Parameter #	New value				
Incpar	Increments a parameter	Use to increase loop iteration value	Step #	Parameter #	Parameter increment value				
Acquire	Acquires data		Save as... ³ 0 = .dax 1 = .tif	# of frames ³	EM gain ³	CCD exposure ³			
Options	Options on or off	0/1 = on/off	Automated laser control	Automated filter wheel	Automated motion control				
Laser	Adjusts laser control	P(n)= power of channel 0-1 corresponding to control slider value	647 channel	561 channel	488 channel	405 channel			
Filter Wheel	Sets angle of filter wheel		Angle value 0 to 360						
ZSCpar	Adjusts z scan parameters	Relative position	Start position (μm)	Stop position (μm)	Increment (μm)	Stage choice 1 = MCL AO	Zscan mode 1= cont. scan	# of scans	# of frames per step
Valve	Sets valve position	Integer values between -8 and +8	0 = initialize value +n = move to port n in clockwise direction -n = move to port n in counter						

			clockwise direction						
Pump	Control pump speed and direction	Uses values - 50 to 50 S>0 is clockwise	Pump speed, S 0.0 is not moving						

¹Refers to another array where you can populate rows with configuration files, the number will correspond to the row number of that array (0-n). ²This number will increase during macro operation. ³If value is not specified it will default to the values previously loaded in CCD control panel on the main page.

Sample Macro Sequences

Example 1:

In this example a loop is created in which the program waits eight seconds then acquires a .dax file. Prior to the loop execution the initial iteration of the loop is set to zero. This process repeats itself for a total of five iterations and stops.

STEP #	Command	P(0)	P(1)	P(2)
0	Init			
1	Setpar	5	1	0
2	Incpar	5	1	1
3	Wait	8		
4	Acquire	0		
5	Goto	2	0	5
6	exit			

Example 2:

In this example a loop is created in which the program waits eight seconds then acquires a .dax file. Prior to the loop execution the initial iteration of the loop is set to zero. This process repeats itself for a total of three iterations and stops. Note while the total iteration value for step #5 is six, only three iterations are executed because it is being incremented by two each loop cycle (Goto P(2)/Incpar P(2) = # of loop iterations).

STEP #	Command	P(0)	P(1)	P(2)
0	Init			
1	Setpar	5	1	0
2	Incpar	5	1	2
3	Wait	8		
4	Acquire	0		
5	Goto	2	0	6
6	exit			

Example 3:

In this example the valve is initialized and then moved to port three in the clockwise direction and then the pump moves at 20 rpm in the counterclockwise direction for 15 seconds at which point it stops pumping and the macro sequence ends.

STEP #	Command	P(0)
0	Init	
1	Valve	0
2	Valve	3
3	Pump	-20
4	Wait	15
5	Pump	0
6	exit	

Example 4:

This is an example commonly used for ultra-low data collection. It consists of a 30 iteration loop in which a movie is acquired followed by a series of flow steps.

STEP #	Command	P(0)	P(1)	P(2)	P(3)	P(4)	P(5)	P(6)
0	Init							
1	Valve	0						
2	Setpar	20	1	0				
3	Incpar	20	1	1				
4	Laser	1	0	0	0	0	0	0
5	Acquire	0	10000	30				
6	Laser	0	0	0	0	0	0	0
7	Valve	4						
8	Pump	-5						
9	Wait	20						
10	Pump	0						
11	Wait	60						
12	Valve	-8						
13	Pump	-20						
14	Wait	30						
15	Pump	0						
16	Valve	3						
17	Pump	-5						
18	Wait	20						
19	Pump	0						
20	Goto	3	0	30				
21	Exit	0						

Turns 647 laser to full power and acquires 10000 frames with an EM gain of 30 before turning laser off

Pumps counterclockwise at 5 rpm for 20 seconds before waiting for 60 seconds. This is used for delivering DNA to the sample and hybridization

Pumps counterclockwise at 20 rpm for 30 seconds. This is used for rinsing with PBS.

Pumps counterclockwise at 5 rpm for 20 seconds. This puts switching buffer on the sample

VITA

Lauren A. Gagnon grew up in the town of Smithfield, Rhode Island where she lived with her parents, Colette and David; twin older brothers, Nicholas and Douglas; and several cats and dogs throughout the years. As a child she loved horseback riding, playing sports, and crushing the dreams of her older brothers such as the time she ruined Santa Claus for them when she was three. She graduated at the top of her high school class in 2009 and moved to Boston, Massachusetts to attend Emmanuel College. There she studied Chemistry and Sociology, receiving her Bachelors of Science in 2013. Shortly after, she moved to Seattle, Washington to pursue her PhD in Chemistry under the mentorship of Dr. Joshua C. Vaughan. While there she met her best friend and now husband, Dmitriy Kapitanov, and adopted her precious ginger cat, Smeagol.