Localization of Dense Clusters of Nanoscale Emitters for Super-resolution Microscopy

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Localization of Dense Clusters of Nanoscale Emitters for Super-resolution Microscopy

by

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A thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Electrical, Computer, and Energy Engineering 2014
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Localization of Dense Clusters of Nanoscale Emitters for Super-resolution Microscopy
written by Anthony J. Barsic
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Date

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Single-Molecule Localization Microscopy is an optical imaging paradigm which provides computational reconstructions of microscopic objects much smaller than the imaging wavelength. Information regarding physical dimensions of small structures arises from precise localization of individual fluorophores attached to targets within the sample. Complete reconstructions require precise localizations for which neighboring emitters must be resolved beforehand. Control of experimental conditions ensures adjacent emitters are not likely to be activated in the same camera frame. As a result, the gain in spatial resolution occurs at the expense of the acquisition time. Unfortunately, this approach necessitates many images to generate a well-sampled reconstruction. The work presented in this Dissertation relaxes the resolution requirements for emitters in spatiotemporal proximity. The methods described herein use experimental, optical, and computational techniques.

Quantum dots are promising for fluorescence experiments due to their brightness and photostability. They exhibit random blinking under constant illumination creating a signal intermittency that is sometimes considered problematic. I present a method for localizing classically unresolved quantum dots by taking advantage of the random, independent nature of the blinking. Experiments confirm the validity of the approach while also showing that nearby quantum dots are indeed independent.

Many experiments require three-dimensional information. A microscope can be modified to transmit precise 3D information through a technique called point spread function (PSF) engineering. The gain in axial precision is achieved at the expense of an increased PSF cross-section at focus, thus exacerbating the problem of emitter overlap. The algorithms presented here exploit the mathematical sparsity of localization microscopy data to identify
otherwise unresolved PSFs, yielding a tenfold enhancement in allowable fluorophore density. The method is applied to localization microscopy data of dye-labeled microtubules resulting in quantitative measurements of the cellular structure.

Localization microscopy is expected to transform biology as the techniques encompassing disparate fields become widespread. This Dissertation also investigates multicolor and 3D super-resolution using original computational and optical tools incorporated into a standard microscope at the BioFrontiers Imaging facility. The system is applied to imaging in the nuclei of virus-infected cells to examine the viral reproductive properties.
Dedication

To the many great teachers who have inspired me to keep seeking.
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4.1 Difficulties with localization in thick samples. The problem of excessive background due to out-of-focus fluorophores in thick samples reduces the localization precision in super-resolution microscopy. Furthermore, drift correction using fiducials is not applicable when the coverslip/sample interface is far from the focal plane. Hence, fiducial-free drift correction methods are required.

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K.1 **Localization Precision Estimates.** Predicted localization precision is plotted over the range of experimentally realizable emitter intensities and background levels.
Chapter 1

Introduction

A variety of tools are available to the modern scientist enabling the inspection of very small objects. The plethora of imaging modalities includes optical microscopy, electron microscopy, atomic force microscopy, and tomography, among others. Optical microscopy can be further sub-divided into categories such as: bright field, dark field, phase microscopy, fluorescence, confocal, near-field, and non-linear techniques [2]. Depending on the object under investigation and the qualities that are of interest, one or more of these modalities may be informative.

Far-field optical microscopy is the appropriate choice for many situations; advantages include speed, ease of implementation, and flexibility. Moreover, light microscopes are affordable and ubiquitous. Light is typically non-invasive, which means optical microscopy can be performed on live cells. An additional advantage of optical microscopy is the ability to easily combine several modalities in a single platform, such as bright field, fluorescence, phase contrast microscopy [3], and various nonlinear modalities (e.g. two-photon excitation [4] or harmonic generation [5]). Fluorescence has proven to be a powerful tool for biological applications. In a complex cellular environment, the ability to specifically observe a particular object of interest and ignore others makes biological imaging experiments tractable. The initial demonstration of immunofluorescence [6] was concerned with observing the accumulation of fluorophore-labeled antibodies in tissues, and even specific cells, that have been
affected by a disease or virus. Subsequent fluorescence experiments explored cell membrane permeability [7], and enabled observation of dynamics in living cells [8]. Understanding of fluorophore characteristics led to developments such as Förster Resonant Energy Transfer microscopy, which can be used for spectroscopically measuring molecular length scales on the order of 1-6nm [9]. Further modifications to fluorescence microscopy, such as epi-fluorescence (in which excitation and detected fluorescence emission fields are counter-propagating), Total Internal Reflection Fluorescence (TIRF) microscopy [10], and confocal microscopy [11] provide images with exceptionally low background.

The advantages of fluorescence microscopy are extensive, but there are many challenges as well. The resolution limit of conventional microscopy restricts the applications because many cellular features of interest are unresolved. Overcoming the resolution limit has been the primary driver of advancements in the field. The imaging speed of fluorescence microscopy is fast enough to capture some live dynamics [8], but there are many processes still out of reach. Furthermore, volumetric imaging requires additional measurements that inhibit imaging speed. The high magnification used in microscopy has the consequence of limiting the field of view, thus hindering studies that examine molecular, cellular, and organismic phenomena in concert. However, the registration and combination of electron microscopy images has in one case resulted in one of the largest images in the world: a 281 gigapixel image of zebrafish embryo [12]. Obviously, the increased effective field of view comes at the cost of measurement time; that particular image took more than 4 days to acquire. Yet another obstacle is the sample limitations necessitated by particular fluorescent probes; the motivation for label-free methods is significant. Some experiments require imaging in thick samples which will cause errors due to aberrations [13]. In the extreme case of turbid media, samples demand approaches that can focus a spot to form an image in the presence of scattering [14].
1.1 Diffraction Limit and Resolution

Optical microscopy has been long thought to have a fundamental resolution limit, as dictated by the diffraction of the light waves that form the image. Ernst Abbe is often credited with first quantifying the diffraction limit:

\[ d = 0.5 \frac{\lambda}{NA} = 0.5 \frac{\lambda}{n \sin \alpha}, \]  

(1.1)

where \( \lambda \) is the wavelength of light, \( NA \) is the numerical aperture, \( n \) is the index of refraction of the material between the object and the objective, and \( \alpha \) is the half-angle of acceptance of the objective [15]. Similarly, there is also an axial resolution limit:

\[ d_z = 2 \frac{\lambda}{NA^2}. \]  

(1.2)

Even with the best objective lenses available (in which diffraction-limited performance is achievable with \( NA = 1.49 \)) and visible light (\( \lambda = 400 - 700 \text{nm} \)), this limit is still approximately 200 – 300 nm in the transverse dimension and 500 nm axially. This is restrictive; much of the objects of interest in biology are one or two orders of magnitude smaller. Clearly, if visible light is to be used for imaging smaller structures, advanced methods are required.

However, there is another resolution criterion formulation that is commonly quoted when discussing the diffraction limit: Rayleigh’s criterion. In this alternative statement, the resolution limit is:

\[ d = 0.61 \frac{\lambda}{NA}. \]  

(1.3)

Again, the diffraction limit is stated as a quantity that depends on the ratio of the wavelength \( \lambda \) and the numerical aperture \( NA \). Although these two criteria only differ by the coefficient in front of the ratio \( \lambda/NA \), it is instructive to contemplate the significance of this coefficient. In the case of the Rayleigh criterion, the coefficient 0.61 comes from the width of the Airy disc, which is the shape of the Point Spread Function (PSF) for a clear circular pupil function. In the Rayleigh criterion, the limit is defined as the minimum separation between two point
sources which still permits the two sources to be identified. The boundary is selected, somewhat arbitrarily, as the case where the first null of the Airy Disc coincides with the maximum of the second point source. An example of the resulting image of two closely-spaced point sources is shown in Fig. 1.1. This spacing results in a dip in the intensity that is $27.5\%$ less than the maximum values.

![Image of two closely-spaced point sources](image.png)

**Figure 1.1: Rayleigh resolution criterion.** Two point sources are deemed to be resolvable if the separation between them is greater than or equal to the distance between the peak and the first null of the Airy Disc in the image of the point sources. The image (a) and cross-section (b) show two point sources separated by exactly the Rayleigh limit. The simulation parameters are $\lambda = 670\text{nm}$, $NA = 1.45$, and a separation of $d = 282\text{nm}$, with a clear circular aperture resulting in an Airy disc PSF.

In light of this description of the Rayleigh criterion, how can we attach a physical interpretation to the Abbe criterion? This is best understood in terms of the modulation transfer function (MTF) of an optical system with a clear circular pupil function [16]. For a circular aperture with a size $2w$, the maximum spatial frequency transmitted through the optical system is:

$$\rho_0 = \frac{w}{\lambda z} = \frac{NA}{\lambda}. \quad (1.4)$$

The spatial frequency $\rho_0$ has units of lines/mm. By inverting this value, we obtain a quantity describing the minimum grating period that can still be viewed with non-zero contrast in
Clearly, both values communicate important insight about the information carried in a conventional imaging system. The fact that these criteria apply to imaging systems in general is what permits us to break these barriers when imaging in a particular modality and the permissible assumptions associated therewith. Both limits depend only on the numerical aperture and the wavelength, but there are many other factors that affect the ability to resolve two point sources. Some of these factors include: the absolute and relative intensities of the emitters, the sources and strength of noise in the system, and the pixelization of the detector (i.e. sampling). Additional information that can be taken advantage of includes knowledge of the optical system (e.g. PSF) and also knowledge about emitters, e.g. size, number/density, coherence, polarization, and temporal behavior, to name a few. Additionally, emitter properties can be controlled through a variety of techniques which exploit nonlinearities in fluorophore excitation. Some techniques do so in a spatially controlled manner [17, 18, 19], while others rely on stochastic readout [20, 21, 22, 23].

Figure 1.2 demonstrates a few examples in which the Rayleigh limit is violated. In the top row, the two emitters are further apart than the Rayleigh limit by 10%, and one emitter is 50% brighter than the other. The two emitters can be resolved in a low-noise situation (Fig. 1.2a), but with higher noise (b), the second emitter is difficult to identify. In the cross-section plots (Fig. 1.2c), there is no dip in intensity between the two emitters. The second row (d-e) displays a situation in which the emitters are closer than the Rayleigh limit by 10%. However, the combination of high spatial sampling, very high signal levels, and low background makes identification of two emitters easier. The cross section in (d) shows a noticeable dip in intensity. The third row shows another situation in which two emitters are closer than the Rayleigh limit—here, the separation is half the Rayleigh limit. In image (f), both emitters are “on” and not easily resolved. Images (g) and (h) show subsequent frames in which one emitter is switched on and the other switched off (denoted by green x’s and blue circles, respectively). Temporal switching allows the individual emitters to be resolved.
Figure 1.2: **Breaking the Rayleigh Limit.** The first row (a-c) depicts a case where two emitters are further apart than the Rayleigh limit. With low noise in a), the emitters can be resolved, but the increased noise in b) inhibits resolution. The cross-sections in c) show no dip between the two emitters for the high noise case from b). The image and cross-section in (d-e) simulate two emitters closer than the diffraction limit; favorable sampling, signal strength, and noise conditions enable resolution. The final example (f-h) demonstrates a case in which two emitters are separated by half the Rayleigh limit. When both are emitting, as in f), they are unresolvable. If the emitters can be switched on (green x’s) and off (blue circles) in two different frames (g,h), resolution can occur temporally.

### 1.2 Single Molecule Localization Microscopy

Single Molecule Localization microscopy (SMLM) is an imaging modality that allows for significant improvement in resolution over the diffraction limit. The power of this technique comes from the following set of assumptions:

- (a) The object consists of many point sources.
- (b) The PSF of the optical system is known.
• (c) The point sources are switched in time such that significant overlap of neighboring images of point sources is avoided.

In fact, these assumptions are what permit sub-diffraction limited resolution in the final example in Fig. 1.2. In order to generate large well-sampled super-resolution images, the process can be conceptually understood as two steps: resolution and localization. Resolution of nearby emitters is enabled by the final assumption in the above list; this can be achieved experimentally in several ways. The initial demonstrations [20, 21, 22, 23] involved fluorophores with a three-state energy landscape:

1. an “on” state, in which the fluorophore emits light when excited

2. a temporary “off” dark state, in which the fluorophore cannot be induced to emit light

3. a permanently off, or “bleached,” state.

A standard fluorophore model, in comparison, does not have state (2). By creating experimental conditions in which a very small percentage (perhaps \( \frac{1}{100} \) of 1%) of the fluorophores are in state (1), and the remainder are in state (2), assumption (c) is valid. Hence, emitters that are closer than the diffraction limit are unlikely to be “on” in the same frame, and thus they have been resolved. By collecting many frames, the nearby emitters are resolved sequentially. This method of achieving super-resolution through time multiplexing can be interpreted as trading temporal resolution for spatial resolution.

The second step in SMLM, namely the localization, occurs in the image processing. Using assumptions (a) and (b), the resolved point sources in each frame can be fitted to a model of the system PSF to high precision. The precision of the fit is limited only by the number of photons detected from the emitter and the noise in the images. In the photon noise-limited case, the relationship between the number of detected photons and localization
precision is:

\[ \langle (\Delta x)^2 \rangle = \frac{s^2 + a^2/12}{N}, \]

(1.5)

where \( \Delta x \) is the localization error, \( \langle \rangle \) is the expectation value, \( s \) is the standard deviation of the PSF, \( N \) is the number of photons detected, and \( a \) is the size of the pixel (where \( a < s \)) [24]. For a given wavelength and optical system, the localization precision is related to \( \frac{1}{\sqrt{N}} \). As an example, the fluorescent dye Alexa 647 emits at 670nm, and has a size of 2.3nm [25]. Green Fluorescent Protein (GFP), a version of which can be used in SMLM experiments, has a similar size of approximately 2.4-by-4.2nm [26]. The PSF has a size on the detector of 549nm (the null-to-null width of the Airy disc when imaged with an NA=1.49 objective, in units of object space). For typical photon and background levels, the transverse precision can be as good as a few nanometers to perhaps tens of nanometers. These relative sizes are shown graphically in Fig. 1.3.

Figure 1.3: Visualization of the relative length scales of point spread functions, localization precision, and fluorescent emitters. To demonstrate the resolution enhancement made possible by localization microscopy, this figure shows an Alexa 647 dye molecule (the small green point in the center), the point spread function when imaged with an NA=1.49 objective (shown in red), and experimentally achievable localization precision of 10nm (blue circle). The scale bar is 100nm.

The entire process of localization microscopy is summarized graphically in Fig. 1.4 using a simulated example. In part (a), the simulated microscopic object is shown in black.
Figure 1.4: **Demonstration of Single Molecule Localization Microscopy.** (a) shows the true object in black. Fluorescence labeling is simulated with randomly-placed green points on the object in (b). A standard fluorescence image is shown in (c). Individual frames are collected, containing only a small number of “on” emitters, as in (d). The combined list of points is plotted in (e), and the rendered super-resolution image is shown in (f). Scale bar: 500nm.

Next, the object is randomly labeled with fluorophores, shown as green points in (b). If this object were to be imaged in a standard fluorescence microscope, with no temporal switching of the fluorophores, the results would appear significantly blurred, as in (c). In the modality of SMLM, many frames are collected, as in (d). In each frame, only a small number of well-resolved fluorophores are detected, and they appear as images of the diffraction-limited PSF on a dark background. Each image of the PSF is then localized to high precision by fitting it to the known shape of the PSF. Tens of thousands of frames may yield hundreds of thousands of points in (x,y) space, which are plotted in (e). Although the scatter plot of these points already resembles the object, such a view does not communicate the uncertainty in localization. Thus, a simple scatter plot tends to suggest higher precision than the true
situation. Typically, the points are used to render a super-resolution reconstruction image, as in (f). In this case, the reconstructed image is rendered by placing a 2-dimensional Gaussian at each point, with a size inversely related to the precision with which that emitter was localized.

Localization microscopy requires temporal switching of fluorophores to enable localization of emitters spaced less than diffraction limit. The two main approaches are PALM (Photo-Activated Localization Microscopy) [20, 22] and STORM (STochastic Optical Reconstruction Microscopy) [21]. PALM uses photo-activatable proteins, such as PA-GFP (photo-activatable green fluorescent protein). PA-GFP possesses the important three-state property mentioned previously. Activation from the dark state to the excitable state is achieved with a 405nm laser. The mechanism of activation is a photoconversion which causes a subtle change in the structure of the chromophore. The subtle change results in a dramatic increase in the absorption of 488nm light; the increase is approximately 100-fold [27]. Once activated, the fluorophore can be excited and localized. The dose of activation light determines the density of activated fluorophores; proper control of the dose results in an average density of less than one activated fluorophore per diffraction-limited spot.

The procedure for photo-switching in STORM is conceptually similar, although the precise mechanism differs. STORM uses fluorescent dyes. Switching can occur in several ways in STORM, but the first implementation used coupled dye pairs to excite only a small fraction of molecules per frame. The cyanine dye “Cy5” is an organic fluorescent molecule that also exhibits a reversible non-fluorescent state. Cy5 is excited in the red (646nm absorption maximum); excitation causes the dye to switch to an off state. A green laser (532nm) reliably converts the dye back into an on state. Furthermore, experiments demonstrate that the presence of a Cy3 molecule in close proximity results in an increased conversion rate [28]. With this observation, Cy5/Cy3 pairs were ready for their own version of a super-resolution experiment with a similar excitation/activation scheme to achieve frames of well-resolved dye molecules [21].
A further development provided the ability to perform STORM without the need for a second dye. This method, called dSTORM (direct-STORM), relies on a “switching buffer” containing β-mercaptoethylamine (MEA) or β-mercaptopethanol (βME) [29]. MEA and βME are the “quenching” molecules; when they bind with a dye molecule, the dye is converted to a dark state [30]. A dose of activation light (e.g. 405nm) induces the dye to release the quencher, thus reverting to a fluorescent state. This change in STORM labeling procedure greatly simplifies the preparation of STORM samples, making the technique accessible to a much wider range of researchers. Rather than requiring custom dual-dye label methods, sample preparation can be performed using a wide range of off-the-shelf dye-labeled antibodies [31].

1.3 3D localization microscopy with an Engineered Point Spread Function

The above description of SMLM provides two-dimensional super-resolution images. Many experiments, however, require three-dimensional (3D) information. A standard PSF carries some information regarding the axial position of a point source, as can be seen in the top row of Fig. 1.5. As the point source deviates from best focus, the image of the PSF blurs. Thus, the axial position can be related to the size of the PSF. This blurring is symmetric about the focus, which results in an undesirable ambiguity regarding which direction from focus the point source is located.

An optical system can be modified in several ways in order to better transmit depth information. A conceptually simple version involves splitting the light onto two separate cameras, one of which is deviated from best focus [32]. The PSF remains the same, but the pair of images with slightly different defocus eliminates the ambiguity described above. One disadvantage of this technique is that in low light experiments, splitting the emission onto two detectors results in a degraded signal-to-noise-ratio (SNR), and exacerbates the effect
Figure 1.5: **Examples of several Point Spread Functions through focus.** The three rows in this figure show the image of a point source with three different imaging systems. The top row (a) shows the standard clear aperture, the middle row (b) shows an astigmatic system, and the bottom row (c) exhibits the Double-Helix Point Spread Function.

of camera noise. Yet another option is to add astigmatism to the system, meaning that the best focus in the two transverse (x,y) dimensions no longer coincides in the same axial plane [33]. Astigmatism can be implemented with a cylindrical lens. The resulting PSF can be seen in the second row (b) of Fig. 1.5. Therefore, the axial position can be estimated by fitting the image of the PSF to a 2D Gaussian function with different widths in each transverse dimension; the ratio of the widths provides an estimate for the axial (z) position.

A standard imaging system can be modified by altering the phase in the pupil plane such that the resulting PSF transmits axial location information more precisely. This process is called Point Spread Function Engineering. An example is the Double Helix Point Spread Function (DH-PSF) [34]. The behavior of the DH-PSF is demonstrated in row (c) of Fig. 1.5. When in focus, the PSF consists of two lobes displaced horizontally; these lobes rotate about each other with defocus. This provides a precise method to estimate the axial location. In comparison with the astigmatic PSF, the DH-PSF provides more consistent precision over a longer axial range [35]. For a description of the optical set-up for generating the DH-PSF,
see Chapter 3, or Ref. [36].

1.4 Resolution and Localization of Overlapping Emitters

The main drawback of localization microscopy is the long time required to collect enough frames to generate a well-sampled super-resolution image. Typical experiments require tens of thousands of frames, which may take anywhere from tens of minutes to more than an hour. Early demonstrations of super-resolution using localization utilized carefully controlled experimental conditions to ensure minimal overlap of neighboring images of PSFs [20, 21, 22]. However, as shown previously, prior knowledge of the sample and optical system permits resolution of overlapping emitters. The primary focus of the body of work presented in this Dissertation is to provide localization algorithms that permit significant overlap of nearby PSF images, and present experimental implementations thereof. An increase in the allowable labeling and activation density in super-resolution experiments results in a decreased data acquisition time, and reduces error in localization when the assumption of well-separated emitters is violated.

The choice of emitter in super-resolution experiments is of paramount importance. Quantum Dots (QDs), for example, exhibit random blinking behavior under constant illumination [37]. This knowledge of the temporal statistics of an emitter has been utilized in several ways. A conceptually straight-forward approach provided super-resolution of two neighboring QDs by classifying frames as containing (a) only one emitter, (b) only the other emitter, (c) both, or (d) none. A decision was made by observing the change in intensity with blinking; the “single on” frames were then fit to a 2D Gaussian function using a least-squares approach [38]. A different technique employed a more sophisticated algorithm from the discipline of blind source separation. Specifically, Independent Component Analysis (ICA) provides the separated images of overlapping QDs using only the assumptions of independence and randomness of the sources; no spatial assumptions were made [39]. An
interesting use of blinking is detailed in [40]. Using an astigmatic system, these researchers are able to achieve excellent localization in 3D by performing analysis not on raw images, but rather on images formed by finding the difference between successive frames. On/off blinking events are made especially apparent in these difference frames. However, this method does not actually resolve overlapping emitters. The approach is relevant for very sparse samples, or for tracking experiments.

A related technique based on statistical fluctuation of emitters, SOFI (Super-resolution Optical Fluctuation Imaging) [41], provides moderately super-resolved images [42]. However, SOFI does not provide the locations of individual molecules or convey three-dimensional (3D) information without scanning, and thus is not directly comparable to localization methods. Similarly, an algorithm employing successive iterations of deconvolution can handle high emitter densities, but does not provide locations of individual emitters [43].

Some samples do not permit the use of QDs due to complications of delivery to or labeling of the object of interest, and must therefore rely on more traditional fluorescent dyes or proteins. Researchers have proposed fitting schemes that allow for a few of these types of emitters generating overlapping images. Perhaps the first fitting algorithm for localization microscopy that applied to the general fluorescence emitter was DAOSTORM [44], which was primarily a case of astronomy software (DAOPHOT [45]) being re-purposed for localization microscopy. In another instance, multiple overlapping emitters are localized sequentially using a Maximum Likelihood Estimator (MLE) approach [46]; this particular scheme addresses the issue of overlapping emitters by performing a predetermined number of iterations on each localized emitter in a cluster to fine-tune the location of each source. The Bayesian Bleaching and Blinking (“3B analysis”) algorithm models the entire data set simultaneously [47]. By viewing the data set holistically, more assumptions regarding emitter behavior can be utilized.

Concepts from the field of compressed sensing were also applied to the problem of localization, resulting in an algorithm that permitted higher densities than previously possible,
albeit only in two dimensions [48]. Although that algorithm used the term “compressed,” the important assumption was the sparsity of the data in feature-space. Claiming the method to be “compressive” is debatable, considering that localization microscopy is highly non-compressive in the standard implementation; thus, this class of techniques is more appropriately referred to as “sparsity-based.” The approach proposed in [48] required prohibitively long computation times; the same group described an improved approach which reduced the computation time by one or two orders of magnitude [49]. The sparsity assumption can also be combined with Bayesian methods, as in the algorithm SSM-BIC (Structured Sparse Model and Bayesian Information Criterion) [50]. In this approach, an initial guess includes potential emitters in any pixel above a threshold. The Bayesian criterion, which compares the image to the reconstruction, is repeatedly computed as the list of potential emitters is deflated. The optimal solution is selected as the estimate with the minimum Bayesian score and the fewest number of emitters. Most of the sparsity-based approaches operate on a pre-defined grid. A recent work addresses this issue by correcting grid-based localization by expressing sub-grid shifts of the PSF as a Taylor approximation of a grid-based PSF and a shifting kernel [51].

The 3D localization methods described in the previous section rely on modifications to the optical system that impart depth information into the image of the PSF. The additional information comes at the cost of an increase in the spatial extent of the PSF. Thus, the need for algorithms that can tolerate overlapping images of the PSF is even more urgent for 3D methods. One such technique is called 3D-DAOSTORM in [52]. As expected, the algorithm is an adaptation of the DAOSTORM/DAOPHOT methods [44, 45]. However, solving the problem in 3D using the DAOSTORM approach is computationally expensive. To ameliorate the computation time, estimated locations of individual emitters are only updated one at a time. Successive iterations address subsequent neighboring emitters, akin to the previously-mentioned Multiple-emitter Fitter Analysis from [46]. A more recent approach combines deconvolution, dictionary-based sparsity assumptions, and Taylor approximations to obtain
continuous estimates of 3D locations of overlapping PSFs [53].

All above-mentioned algorithms aim to reduce the acquisition time of SMLM (and thus improve the temporal resolution) by permitting precise localization of overlapping PSF images. An orthogonal approach to combat the problem of long acquisition times takes advantage of the very high frame rates of the newest generation of sCMOS (scientific Complementary Metal Oxide Semiconductor) cameras [54]. In choosing to use a CMOS device, these researchers have departed from the typical camera choice of an EMCCD (Electron-Multiplying Charge-Coupled Device). EMCCDs are the standard camera for low-light level applications such as fluorescence microscopy and SMLM in particular. Of course, the number of localizations necessary to form a useful super-resolution image depends highly on the object under investigation. Object-dependence makes any objective claim of the absolute time required to acquire data for a super-resolution reconstruction somewhat tenuous. Regardless, higher frame rates mean faster imaging. However, the high frame rates also require high laser intensities, which are not acceptable in some experiments.

1.5 Outline of the Dissertation

The work presented in this Dissertation addresses the problem of localizing dense scenes of emitters for the purpose of super-resolution microscopy. The first section describes a technique utilizing blinking quantum dots for super-resolution. The next chapter reports on a class of sparsity-based methods. The following chapter of this Dissertation illustrates the details, challenges, and solutions to making such methods available to the general biology researcher. Lastly, an alternative method for 3D localization is demonstrated.

Chapter 2 addresses the super-resolution approach using Quantum Dots. As previously described, QDs exhibit blinking behavior when illuminated with a constant intensity. Knowledge of the temporal characteristics provides the opportunity for several super-resolution methods [38, 39, 41, 42, 40]. I propose a super-resolution technique for dense clusters of
blinking emitters. The method relies on two basic assumptions: the emitters are statistically independent and a model of the imaging system is known. We numerically analyze the performance limits of the method as a function of emitter density and noise level. Numerical simulations show that five closely packed emitters can be resolved and localized to a precision of 17 nm. The experimental resolution of five quantum dots located within a diffraction-limited spot confirms the applicability of this approach. Statistical tests validate the independence of our quantum dots separated by nanoscale distances [55].

The details of the sparsity-based methods are found in Chapter 3. The localization scheme illustrated therein can identify several overlapping images of a 3D PSF while maintaining high localization precision. In fact, the allowable labeling density is an order of magnitude higher than previous implementations of the Double Helix, potentially resulting in a similar acceleration in the data collection time of 3D SMLM. The problem of estimation with a 3D PSF is significantly more complex than the 2D case. For this reason, two different estimation schemes are investigated in regards to the number of recovered molecules, the localization accuracy, and computation time. A hybrid method combines the strengths of the two approaches. The methods are then applied to experimental super-resolution data of tubulin labeled with Alexa-647 and Alexa-488 dyes and imaged with a Double Helix microscope. Analysis of the reconstruction yields a quantitative measurement of the radius of the microtubule, which has a size of approximately $\frac{\lambda}{20}$ (where $\lambda$ is the emission wavelength of Alexa-647). Additional simulations verify that these sparsity-based methods can be applied to other 3D PSFs, such as the astigmatic PSF. The astigmatic and Double Helix PSFs are compared as a function of emitter density and sample thickness [56, 57].

In Chapter 4, I present multi-color super-resolution results from a thick sample. The sample thickness is several times the depth of field, which results in high background and reduced localization precision. The successful two-color results were enabled by a combination of proper fluorophore selection and optical system layout in addition to computational tools. Specifically, fast data examination tools allowed for effective testing of combinations of fluo-
rophores. Thick samples also present a problem regarding drift correction; since the coverslip interface is far from focus, fiducial markers are not an option. I implement a label-free drift-correction method based on repeated dye photoswitching. The resulting super-resolution images depict viral reproduction centers within the nucleus of a mammalian cell. These structures have been studied with standard fluorescence and confocal microscopy and also electron microscopy [58]; the results shown here bridge the gap between those modalities. Additional testing evaluates the possibility of performing two-color 3D imaging by modifying a commercial system.
Chapter 2

Super-resolution of Quantum Dots using Blinking Statistics

Conventional optical microscopy has a fundamental resolution limit determined by diffraction. However, the resolution paradigm is changed if one can assume the object consists of a collection of point-like sources whose emission can be controlled, as is the case in single-molecule localization microscopy. Recently proposed super-resolution fluorescence microscopy techniques rely on the ability to temporally resolve closely-spaced emitters via photoactivation or photoswitching [21, 20, 22]. The data acquisition speed is limited because only a sparse subset of emitters is active at any given time to avoid overlap of their images.

Several techniques to resolve emitters with overlapping images have been proposed lately. Some apply prior spatial information using parallel fitting algorithms [46, 44], sparse reconstructions [48], or Bayesian estimation [50, 47]. Other methods utilize temporal intensity fluctuations of the emitters [39, 38, 41, 59, 60]. However, to achieve high performance, it is critical to employ all available prior information. Therefore, in this Dissertation, we propose a method that takes advantage of both spatial and temporal information to resolve and precisely localize many overlapping emitters. We first introduce the model, then present simulations to analyze the performance, and follow with an experimental validation of super-
resolution using Quantum Dots (QDs). Finally, we investigate the statistical independence of the QD emissions.

2.1 Quantum Dots

A Quantum Dot (QD) is a small sphere of semiconductor crystal. The size is so small that the behavior resembles a 3D “quantum well”, hence the name. The diameter of a QD influences the discretized band structure of the system. The discretization means a QD has a characteristic emission wavelength when an exciton recombines; the diameter of QD determines emission wavelength.

Quantum dots exhibit many favorable characteristics that make them excellent choices as fluorescent probes. They are very bright and photostable, meaning they can be imaged for long periods of time before they bleach. The absorption and emission bands are well-separated, which makes filtering much easier. Furthermore, the wide range of emission wavelengths can all be excited at a single wavelength, greatly simplifying the optical system for multi-color experiments [61]. The work presented here uses commercially available CdSe dots from Invitrogen, $\lambda = 525\text{nm}$ ($d_5 \approx 15\text{ or } 20\text{nm}$).

The most notable characteristic of QDs is their peculiar “blinking” under constant illumination [62, 37]. See Fig. 2.1 for an example of the blinking behavior. For tracking experiments, the intermittent signal makes following a single QD over time much more difficult.

2.2 Blind Source Separation as Super-resolution

Blind source separation is a class of techniques for recovering the original set of signals that have been mixed onto a set of detectors. In order for signals to be differentiated, each signal must be sensed by the detectors with different coefficients. Other relevant assumptions are linearity in mixing onto detectors and that the number of detectors must equal or exceed
Figure 2.1: **Quantum dot blinking behavior.** The image is a frame from experimental data of quantum dots on a coverslip. The inset plots show the temporal intensity signal of the indicated pixels in the scene. In this case, the optical system used the double-helix PSF.

the number of signals.

### 2.2.1 Model

The method assumes a video of an unknown number $N$ of fixed emitters with statistically independent intensity fluctuations. The imaging system response is known (theoretically or experimentally). For instance, a common assumption is that of a linear shift-invariant diffraction-limited system. Our model allows for background noise and additive white Gaussian noise. While noise in low light level systems is Poissonian, at even moderately low signal levels it approaches Gaussian statistics.

### 2.2.2 Independent Component Analysis

Independent Component Analysis (ICA) is an approach for performing blind source separation. The main requirement is that the sources must be statistically independent. The first step of ICA is to perform Principal Component Analysis (PCA), which is equivalent to performing Singular Value Decomposition (SVD). To conceptualize ICA and PCA, the video of images is best visualized as a set of points in N-dimensional space, where N is the number of pixels in the image. PCA is simply a rotation of the data such that each
new dimension (principal component) contains the maximum variation. ICA is then a re-mixing of these PCs such that the time signal of each is independent. The primary difference between implementations of ICA is the independence metric. Possible metrics are mutual information, negentropy, correlation-based metrics, and many others.

2.3 ICARUS Algorithm

Two steps are involved while imposing constraints in the temporal and spatial domains. In the first step, we perform blind source separation using Independent Component Analysis (ICA) [63]. ICA decomposes the data into a set of variables with maximally non-Gaussian probability distribution functions. In principle, these variables correspond to the images of the independently blinking QDs in the scene [39].

Unfortunately, the ICA algorithm has one major shortcoming—to correctly resolve the emitters, one must know the true number of emitters present, which is obviously not known \textit{a priori} in an experiment. This would be a difficult enough hurdle to preclude the method from being implemented in any nontrivial situation. We address this problem by incorporating a spatial analysis. After using ICA to generate a family of possible solutions, we score them according to their spatial characteristics and select the best solution. Therefore, we refer to this algorithm as ICARUS (Independent Component Analysis for Resolution Using Spatial information).

In order to explain the spatial component of ICARUS, we present a numerical simulation. Figure 2.2 a) shows the average of a 500-frame data vector generated numerically with four blinking emitters (the locations are marked with x’s). Background noise is added with a Signal to Background Ratio (SBR) of three; shot noise is also incorporated. ICA is implemented using the fastICA package [63]. FastICA’s inputs include the requested number of components $n$ and the output is the set of $n$ independent components. As $n$ increases, the successive outputs form a pyramid of solutions as in Fig. 2.2 b). Each row from this figure
is a potential solution.

![Simulation of ICARUS algorithm.](image)

Figure 2.2: Simulation of ICARUS algorithm. (a) Average of the simulated video with the centers of the sources marked with x’s (scale bar: 100nm). (b) Family of potential solutions for different runs of ICA with different number $n$ of emitters. (c) Scores of the rows from (b) plotted as a function of $n$.

By examining these solutions, a clear pattern emerges: once the number of emitters is overestimated, spurious results that do not resemble the Point Spread Function (PSF) are returned. ICARUS exploits this tendency to estimate the number of emitters. Each potential solution is given a score $S_n$ that is the largest mean squared error of any one of the components as compared to the theoretical PSF:

$$S_n = \max_i \left( \frac{1}{P} \sum_{p=1}^P |\mathcal{F}\{IC_i\}_p - \mathcal{F}\{PSF\}_p|^2 \right),$$

where $\mathcal{F}\{IC_i\}_p$ is the $p^{th}$ pixel of the Fourier transform of the $i^{th}$ independent component, and $\mathcal{F}\{PSF\}_p$ is the Fourier transform of the ideal PSF. $P$ is the total number of pixels.
The calculation is performed in Fourier space to avoid the effect of lateral shifts. The scores for our simulation are shown in Fig. 2.2 c).

One might initially assume that the best estimate of the number of emitters in the scene (\( \hat{N} \)) corresponds to the minimum score. The simulation clearly demonstrates why this is inaccurate. Here, two of the emitters are 20nm apart, and the candidate solution \( n = 3 \) has a component that is the sum of these two emitters. This superposition still closely resembles the ideal PSF and has a good score. In fact, due to the presence of noise, \( S_3 \) is actually lower than \( S_4 \). Similarly, the scores for \( n = 1 \) and \( n = 2 \) are also low. These low scores are due to super-position of emitters or complete omission of an emitter that might be dimmer or less active in its blinking behavior. To avoid an underestimation such as this, a better estimate is the maximum of the forward difference:

\[
\hat{N} = \arg \max_n (S_{n+1} - S_n). \tag{2.2}
\]

In other words, this estimate returns the result immediately preceding the largest increase in the scores, which is a more robust estimate; i.e. overestimation is a more detectable event than correct estimation.

### 2.4 Monte Carlo Simulations

#### 2.4.1 Density Limitations

We perform Monte Carlo simulations to investigate the performance of ICARUS. For each number of emitters from one to seven, we generate a random set of unresolved locations; these emitters have a random bimodal (on/off) blinking behavior with background and shot noise included. Subsequently, the ICARUS algorithm estimates the number of emitters and their locations, and repeats 500 times for each number of emitters and SBR. Although the theoretical maximum number of emitters that can be resolved is equal to the number of pixels, practical results are limited by experimental conditions such as noise and signal lev-
els, spot size, observation time (number of frames), and blinking characteristics. Figure 2.3 a) demonstrates that ICARUS can reliably resolve five emitters within a diffraction-limited spot with typical experimental SBRs. At higher densities, the number of emitters is often underestimated. Furthermore, these super-resolved emitters can be localized to a high precision, as demonstrated in Fig. 2.3 b). Localization precision depends on the noise and emitter density, but typical experimental noise levels allow localization as good as 17nm when five emitters are located within a diffraction-limited spot. An interesting question relates to the number of frames required to attain reliable solutions. Figure 2.3 c) suggests that under typical conditions (four emitters in the scene and SBR=3), a few hundred frames are sufficient.

Figure 2.3: Monte Carlo simulations for varying numbers of emitters and noise levels. Error bars show the standard deviation of 500 repeated simulations. ICARUS's ability to achieve super-resolution is shown in (a) and super-localization precision levels are shown in (b). The effect of increasing the number of frames in the video is displayed in (c). The correct number of emitters is four.
2.5 Experimental Results

2.5.1 Experimental System

In experiments, a 405nm diode laser excites the QDs in a fluorescence microscope (1.3 NA 100x Zeiss objective, 100mm tube lens, 62.5x magnification, Hamamatsu Orca-Flash 2.8 CMOS camera), providing emission at 525nm. This system slightly over-samples, with 3.5 pixels across the full-width at half-maximum of the PSF. A test sample composed of QDs scattered across a cover slip was imaged for 500 frames with an exposure time of 200ms per frame, giving a total acquisition time of less than two minutes.

2.5.2 Experimental Results

The results of ICARUS on a 14x14 pixel scene are shown below. In the normal fluorescence image there are no clearly-resolved emitters, but the ICARUS analysis, based on the maximization of the forward-difference (Eq. 2.2), suggests there are five. Figure 2.4 shows the average of the video, the pyramid of potential results, and the scores. The distance between neighboring emitters is between 85 and 230nm in all cases, and the furthest distance between two emitters is 420nm. For comparison, the diffraction-limited spot is 493nm in diameter. Therefore, all five emitters have been (super-)resolved.

2.6 Independence Tests

In what follows we validate the assumption of independence of QD emissions. This is particularly important because it has been noted that QDs can couple, producing correlated emissions, especially when they are in close proximity [64]. In order to establish independence, we use two statistical measures, namely a bunching test and mutual information (MI).

First, we implement a photon bunching test as reported in Ref. [64] (bunching would
Figure 2.4: Experimental results. (a) Shows the average of the 500-frame video of blinking QDs; the estimated locations of the five emitters are shown as x’s (scale bar: 100nm). (b) The pyramid of potential solutions. (c) Scores of 100 repeated attempts of the ICARUS algorithm on the same set of experimental data. The bold dashed red line is the average of all attempts.

suggest the QDs emit in unison and are not independent). The bunching test is a statistical statement made about two time signals. First, the background is subtracted from the signals and the cross-correlation function $g(\tau)$ computed. A mean cross-correlation value $\bar{g}$ is defined as the mean of the five points to both sides of $g(\tau = 0)$. Similarly, the standard deviation $\sigma$ of the same ten data points is calculated. The pair of signals is considered “bunched” if the cross-correlation function has a central peak that exceeds the surrounding points by more than one standard deviation, i.e. $g(0) \geq \bar{g} + \sigma$.

In Ref. [64], the emitters were clearly resolved, so the pixel containing the center of the emitter’s PSF receives no light from the nearby QD. In our case, the QDs are closer than the
classical diffraction limit. If we select the five pixels that contain the centers of the emitters and compare all ten pairings, we find significant correlation at distances less than 200nm. However, the reason for this behavior is that the center pixels of two emitters closer than 200nm receive contributions from both emitters. Alternatively if we select pixels that are shifted off-center such that the dominant portion of their signal belongs to only one QD, we will observe no correlation even for QDs less than 100nm apart. An example pair is shown in Fig. 2.5, along with the correlation functions for the center and off-center pixels.

Figure 2.5: Mean data image and bunching test. The estimated locations of the emitters are superimposed on the mean of the dataset in (a). The circles indicate the width of the nulls and the full width half maximum of the airy disc (scale bar: 100nm). The squares indicate the pixels used to generate the cross-correlation functions shown in (b). By applying the bunching test to these functions, it can be concluded that even these closely-spaced QDs are not bunched.

While Ref. [64] reports bunching for QD separations under 1.1µm, our QDs show a different behavior, which is not surprising considering the characteristics of QDs depend on
factors such as their environment, preparation method, and emission wavelength.

Statistical independence of two signals is the condition that knowledge of one signal does not provide any information about the other. In other words, the joint Probability Distribution Function (PDF) must factorize to the product of the marginal PDFs of the individual signals. It should be noted that lack of correlation is not sufficient to guarantee independence. Hence, we calculate the mutual information between each pair of QDs. Calculations of MI are directly related to the definition of independence, so a low degree of MI suffices to prove independence [63]. In our calculations, the MI score is normalized so that 0 corresponds to independence and 1 to complete dependence.

To apply this criterion to our data, we select the time signals of the pixels that contain the centers of the five emitters (as estimated by ICARUS). Because we do not have access to the actual PDFs, we estimate them by binning the data. This step introduces a binning error; i.e. even truly independent random variables will have a non-zero MI when they are calculated from binned estimates of a limited number of samples. Simulations of independent random variables with the same number of samples and identical binning procedure yield an average MI score of 0.088, which is indicated as a dashed line in Fig. 2.6. See Appx. B for a more detailed explanation of the threshold. If two signals have a MI score near or below this value, they can be considered independent. By comparing the signals of the central pixels of the QD PSFs, it can be seen from the plot that QDs spaced further than 250nm apart are clearly independent. At closer separations, the MI score suggests a degree of dependence (see the circles in Fig. 2.6). However, this dependence is an artifact of the mixing of two independent signals due to the extent of the PSF. If we use off-center pixels as previously described, we obtain the adjusted MI values indicated as triangles in Fig. 2.6. These signals are independent.

To support our claim that the non-zero MI scores described above are a result of the mixing of independent signals, we perform the same MI calculation on an ideal, noiseless dataset. This dataset is constructed from the outputs of ICARUS (i.e. the emitter images
and time signals). The pure time signals provided by ICARUS are inherently independent. Once the signals are mixed onto the simulated pixels, the degree of MI increases for QDs spaced closer than 250nm (see the x’s in Fig. 2.6). As indicated in the figure, even the most closely spaced QDs in the experimental data have MI scores below the values for completely independent but unresolved emitters from the ideal dataset. The results of these MI calculations indicate that all the QDs observed in this experiment are independent.

![Figure 2.6: Mutual Information (MI) test for all pairings of the five QDs. The MI scores for the center pixels of QDs closer than 250nm suggest the possibility of dependence (circles). However, when shifted off-center pixels are compared (triangles), it is clear that the dependence is only due to overlap of the PSFs and is not caused by QD signal dependence. This is further supported by the evidence that truly independent QDs will yield high MI scores when placed close together (x’s).](image)

2.7 ICARUS in 3D

The spatial aspect of the scoring in ICARUS relies on knowing the shape of the PSF. The advantage of this approach is that it can be applied to any alternate PSF, as long as a model can be made for use in the fitting. Here, I demonstrate the results of ICARUS when applied to 3D data exhibiting the DH-PSF. The two PSFs are well resolved and do not present a particularly challenging piece of data for ICARUS; they are merely meant as
a demonstration.

Figure 2.7: **ICARUS in 3D.** Example of ICARUS with 3D data in a system with the DH-PSF. The results of ICARUS analysis provide the individual images of the two DH-PSFs.

### 2.8 Discussion and Conclusion

Interestingly, fastICA does not return the same results every time. With more emitters and tighter spacing among them, the solution space is more poorly conditioned and incorrect solutions are more likely. This problem is ameliorated by increasing the number of frames, or by increasing the frame integration time to improve the signal level. These same trade-offs apply to the localization precision; higher emitter density, higher noise levels, and fewer frames will worsen the results. This behavior is obvious by examining the plot of the scores from 100 iterations of ICARUS, as shown in Fig. 2.4(c). Although the scores differ between subsequent iterations, ICARUS returns an estimate of five emitters in 66% of the cases. Additionally, the average score plot clearly suggests there are five emitters. The reliability of the estimation is therefore increased by repetition of ICARUS.

The analysis presented here has only been applied to small sections of data. In order to generate larger images, the data could be divided into small windows of, for example, 16x16 pixels. On a single-core desktop machine, a full run of ICARUS on this scale takes one to two seconds. This means one could expect to compute a large image (1024 pixels on a side) in one to two hours, although this time could be significantly reduced with parallel computing. Alternatively, performing ICARUS on large matrices is very computationally intensive; each
fastICA takes longer, and ICARUS has to iterate through more potential solutions (higher $n$). Note that lower noise levels and fewer emitters yield faster calculations.

The advantages of ICARUS are numerous. In particular, it can be implemented with a standard fluorescence microscope. Although other fluorophores could be used, QDs are attractive due to their photostability, high photon output, and versatile spectral characteristics. The only requirement is that the emitters have an independent and non-Gaussian emission characteristic. In a different experimental setting, other scoring criteria could be used to take advantage of prior knowledge, and multiple criteria could even be combined into a merit function to strengthen the estimation. Examples of potentially useful prior knowledge are non-negative constraints, QD blinking statistics, and spectral characteristics. Multiple potential solutions could also be included according to Bayesian probabilities. Furthermore, ICARUS could be extended to three-dimensional super-resolution imaging using modified PSFs [33, 32, 34].

In conclusion, we demonstrated a super-resolution technique capable of resolving dense clusters of nanometric emitters and analyzed the fundamental trade-offs. The simulations validate the method, showing the ability to resolve emitters that would normally be unresolved. Experimental data shows super-resolution well beyond the diffraction limit. The independence tests we presented validate the basic ICARUS assumptions and show it can be applied to close QDs to achieve super-resolution. Interestingly, the outcome also suggests that further research is needed to investigate correlations of QD emissions at sub-diffraction limited distances.
Chapter 3

Sparse Reconstructions for the Resolution of Dense Clusters of Emitters

When a single molecule is detected in a wide-field microscope, the image approximates the point spread function of the system. However, as the distribution of molecules becomes denser and their images begin to overlap, existing solutions to determine the number of molecules present and their precise three-dimensional locations can tolerate little to no overlap. We propose a localization scheme that can identify several overlapping molecule images while maintaining high localization precision. A solution to this problem involving matched optical and digital techniques, as here proposed, can substantially increase the allowable labeling density and accelerate the data collection time of single-molecule localization microscopy by more than one order of magnitude.

In single-molecule localization microscopy [20, 21, 22], sparse sets of emitters are localized by identifying well separated single-molecule images and fitting them to high precision, thereby achieving resolution better than the diffraction limit. Similar problems appear in many biological and biophysical experiments where two or more molecules need to be resolved or their distance estimated [65, 66, 67]. Localization precision can be much better than the diffraction limit, depending on the number of photons detected from the emitter and noise conditions [68]. Lately, photoswitching, photoactivation, and other mechanisms
were proposed and developed to overcome the problem of overlapping molecule images in a time sequential form [20, 21, 22, 23]. The trade-off for super-resolution in these methods is a slower acquisition rate—typically, tens of thousands of frames are collected and processed to generate a single super-resolution image. To ameliorate this problem, researchers have proposed fitting schemes that allow for a few emitters generating overlapping images [46, 47, 48, 52, 55]. Unfortunately, all methods reported so far are limited to two-dimensional imaging [46, 47, 48, 55] or provide a modest increase in emitter density [52]. A related technique based on statistical fluctuation of emitters, SOFI [41], provides moderately super-resolved images [42]. However, SOFI does not provide the locations of individual molecules or convey three-dimensional (3D) information without scanning.

After the work presented here was accepted for publication, another high-density 3D method using sparsity assumptions was proposed [53]. Building from their previous 2D work [51], the authors extended the concepts to 3D by using a combination of astigmatic and biplane PSFs. Although their method achieves good performance at high densities, the choice of 3D PSF limits the useful axial range to less than 1 \( \mu m \).

### 3.1 Algorithms for 3D Localization

Three-dimensional information is required for complete understanding of many biological structures and phenomena. However, 3D localization microscopy suffers from poor temporal resolution. The ability to obtain 3D localization information from dense molecule arrays could enable faster data acquisition and addresses a fundamental problem in 3D imaging. In this Dissertation, we investigate methods to increase the allowable labeling density, namely finding the number and 3D locations of clustered emitters from a single image. The experimental demonstration of the technique in biological samples opens up new opportunities to acquire quantitative information about single molecules and other emitters that remain unresolved in three dimensions with conventional methods. We use microtubules to
demonstrate the ability to measure the full 3D shape of intracellular structures. In particular, we measure the radius of an antibody labeled microtubule while detecting only about 600 photons per emitter. The methods also enable faster acquisition times for 3D single-molecule localization microscopy, which is critical for live-cell super-resolution imaging. Furthermore, the technique is applicable in other areas such as tracking of multiple particles or 3D surface characterization.

Several techniques can encode depth information onto a two-dimensional image by utilizing an engineered PSF [32, 33, 34, 69]. Without losing generality, we chose the double-helix (DH) PSF because of its inherent precision and depth of field advantages [34]. Accordingly, a single emitter in the focal plane generates an image with two horizontally displaced lobes. The transverse location of the emitter is related to the center of the two lobes, and the axial location is encoded in the orientation of the lobes [70]. For illustration, a few dictionary elements for a DH-PSF system are shown in Fig. 3.1. Each dictionary element contains the 2D cross-section of the PSF corresponding to a different discrete emitter location in the full 3D space. Note that the methods demonstrated here can be applied to any 3D PSF and are not limited to the DH-PSF. However, the particular PSF structure can become a significant factor in the overall performance. Section 3.3.2 presents a demonstration of these methods with an astigmatic PSF and a comparison with the DH-PSF.

Regardless of the optical method for transmitting 3D information, the localization algorithm must now estimate the axial position in addition to the transverse position. Searching a 3D space for a solution is a much more computationally intensive task, and algorithms must take this into account. A good algorithm for the 2D case might not be advisable (or even possible) for the 3D case.
Figure 3.1: **Example of elements of an overcomplete dictionary for a 3D PSF.** These images show a selection of dictionary elements for a Double-Helix system for a few different locations in x and z.

## 3.2 Theory of Sparse Reconstructions

In what follows we emphasize the distinction between the image generated by an emitter, such as a single-molecule, and the point spread function (PSF). While the former depends on the emission pattern of the emitter, noise, sample induced aberrations, and the detector array, the latter is only a function of the optical imaging system.

The key observation behind the methods proposed here is the fact that raw images in single-molecule localization microscopy are a combination of sparse (possibly overlapping) molecule images and noise from different sources. This raw image can be efficiently represented with a dictionary consisting of the images of transversely and longitudinally shifted point emitters. A dictionary is a set of vectors that spans the space of possible images. In addition, the 3D PSF can be engineered to facilitate the resolution of dense emitters.

The method for resolving and localizing 3D clusters of single molecules involves a combination of optical and digital techniques: (a) imaging the sample with a proper 3D PSF
imaging system; (b) creating a model of the system via experimental measurements, theoretical calculations, or a combination of both; (c) establishing a dictionary composed of the image of a point source for different locations in a dense 3D grid; (d) solving the estimation problem of determining the coefficients of the dictionary elements that best represent the data. Once the non-zero coefficients are known, the number of molecules and their locations and brightnesses can be determined.

3.2.1 Dictionary Considerations

Dictionaries provide alternate representations to the pixel-based image; i.e. a set of coefficients describing the degree to which each dictionary element is present in the image. Interestingly, a scene that appears dense to our eyes (contains numerous overlapping images) may be sparse in a properly chosen dictionary. Sparse means the image can be expressed by a number of coefficients $K$ that is significantly smaller than the number of pixels used in the scene $N$ ($K << N$). We note that the most efficient representation of a scene with overlapping single-molecule images contains a single coefficient for each emitter in the scene. Since each coefficient in the solution corresponds to an emitter, they are intrinsically resolved, and the coefficients are easily converted to locations and photon counts.

The most important design parameter for a dictionary is the step size between adjacent elements. Small step sizes are desirable so that the localization precision is not limited by step size. Conversely, smaller steps require more elements, hence a more computationally intensive reconstruction. Typical imaging system designs and desired localization precision necessitate sub-pixel steps. Furthermore, the dictionary extends along the axial direction. These two factors mean we require dictionaries that are overcomplete, i.e. there are more elements $D$ in the dictionary than there are pixels per image ($D > N$). Table 3.1 shows the size of the dictionary for various step sizes.

For the dictionaries used in the methods discussed in this chapter, there are a few important guidelines to follow. The topics relevant to dictionary generation that are dis-
Table 3.1: **Dictionary Scaling.** This table shows the typical dictionary sizes for 3D localization using the sparsity-based techniques described here. The top row is a coarse dictionary, and the successive rows represent progressively finer dictionaries.

<table>
<thead>
<tr>
<th></th>
<th>x step (nm)</th>
<th>y step (nm)</th>
<th>z step (nm)</th>
<th>number of elements</th>
<th>Size (MB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarser</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>5,324</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>40</td>
<td>80</td>
<td>21,296</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20</td>
<td>40</td>
<td>170,368</td>
<td>88.9</td>
</tr>
<tr>
<td>Finer</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>1.27 million</td>
<td>678</td>
</tr>
</tbody>
</table>

Discussed in this section are: transverse step size, axial step size, amplitude scaling of dictionary elements, and the use of Look-Up Tables (LUTs) in localization.

All methods discussed in this Dissertation utilize a dictionary that consists of the 2D PSF corresponding to lateral and axial shifted locations of the point source. If the system is shift-invariant, for a given depth, all 2D PSFs are equal except for a transverse shift. The dictionaries are generated by first selecting a 3D window size and making a list of discrete 3D locations to fill this volume. A model is then used to generate the corresponding image for each point source location in the dictionary. The model may be either an ideal PSF, or it may be the result of calibration data. The step size of the locations in the dictionary determines the localization precision, so the natural tendency is to make the step size very fine. A wise choice would be to relate the step size to the limit of localization precision achievable in a given system, as calculated by the Cramer-Rao Lower Bound (CRLB) [35]. The effective pixel size (physical pixel size divided by magnification) in single-molecule detection systems is typically designed to sample the PSF at approximately Nyquist sampling, which results in effective pixel sizes on the order of 160\(\text{nm}\) in our experiments. The CRLB, on the other hand, can be significantly lower (typically 10 to 20\(\text{nm}\)). As a result, the dictionary requires sub-pixel shifts. Additionally, since the extent of the PSF is significantly larger than the step size, there is a high degree of similarity between adjacent dictionary elements. Hence, by definition, the dictionary is coherent [71]. Thus, finding the solution for the representation
of a scene using such a dictionary is a more difficult problem than if we were to use an orthonormal basis.

Furthermore, the requirement for 3D information necessitates many dictionary elements at the same transverse location. The DH-PSF enables an extended depth of field (DOF) as compared to a standard aperture by approximately a factor of two. In our experiments using a 1.45 NA objective, the DOF with a DH-PSF is approximately \( 2\mu m \). However, since the CRLB is larger in the axial dimension than in the transverse dimensions, the axial step size can be also slightly larger than the transverse step size.

Because the dictionary contains discrete locations, the results will have a quantization error. Even if the localization algorithm returns the correct dictionary element for every emitter in a scene, there will still be a distribution of errors spanning a range between \(-d/2\) to \(+d/2\), where \(d\) is the step size. The standard deviation of a uniform distribution with a range of \(d\) is \(d/\sqrt{12}\). We choose to use standard deviation to characterize the error because it can be directly compared to the square root of the CRLB. For consistency, we also use standard deviation to quantify the magnitude of the error in simulations. The plots in Fig. 3.2 show the localization precision as a function of the dictionary step size. The theoretical limit of the standard deviation of localization is determined by experimental conditions such as signal level, noise level, wavelength, numerical aperture, and pixel size. Discrete dictionaries also limit the best achievable precision; the standard deviation of localization precision can never be better than the step size times \(1/\sqrt{12}\). This limit is shown as a dotted red line in Fig. 3.2. There are several important conclusions to be drawn from this plot. First, the algorithms do indeed perform to the limit of the dictionary when the quantization of the solution space is the limiting factor. Related to this observation is the fact that one can always tell if the dictionary step size is too coarse; in such cases, the localization precision will be approximately equal to the limit imposed by the dictionary quantization. Lastly, in many experiments, the localization precision can be estimated beforehand based on system parameters and signal and noise levels. Therefore, one can pick the level of dictionary step
sizes that will not induce significant additional localization error.

Figure 3.2: **Quantization error due to discrete dictionaries.** Simulations of randomly placed point sources provide insight into the effect of discrete dictionary step sizes. The standard deviation of the error in localization precision for 500 frames is plotted here for four different signal levels. The theoretical limit is shown as a dashed red line. The precision matches the theoretical limit for coarse dictionaries; as the step size decreases, localization precision is limited by other factors. Therefore, it is possible to use a fine enough dictionary such that the discretization is not a problem.

The important output of our sparsity-based algorithms is not the reconstructed image, but the indices of the significant coefficients that make up the reconstruction. A high coefficient for a particular dictionary element means there is a bright emitter at that location. Each dictionary carries with it a LUT to convert the index of a dictionary element to a physical location in \((x, y, z)\) coordinates. The LUT is a \(D \times 3\) matrix, where \(D\) is the number of dictionary elements. There are three columns because the solution space is 3D. Each row contains the \((x, y, z)\) values describing the location of an emitter that would generate the PSF in the corresponding index of the dictionary. The LUT is easily generated alongside the dictionary, and provides a simple way to obtain a physical interpretation of the coefficients in the solution.

Each element of the dictionary is normalized such that its L2 norm is unity. As a result, the product of the coefficient and the dictionary element matches the intensity
of that component in the image. The corresponding number of photons is calculated by multiplying the coefficient with the L1 norm of that particular basis element. The photon count calculation is correct only after the data has been converted from camera counts to photons. A matrix containing the L1 norms for each dictionary element is calculated beforehand and stored with the dictionary for fast conversion from coefficient values to photon counts. When performing localization, it is necessary to calculate photon counts frequently, since one of the stopping criteria for the iterative method requires knowledge of the number of photons (stopping criteria are discussed in the following section).

3.2.2 Estimation using Matching Pursuit

If the dictionary were an orthonormal basis set, the coefficients for representing the image could be found very easily: they would be the inner product of the image and each individual dictionary element. However, the use of small sub-pixel shifts means adjacent dictionary elements have a high degree of similarity; they are not orthogonal. Therefore, the coefficients must be found in a different manner. The two methods we propose to solve the estimation problem are Matching Pursuit (MP) and Convex Optimization (CO). These two approaches are described in this section.

To solve the coefficient estimation problem, we investigate two methods that are representative of large classes of solvers. The first method is Matching Pursuit (MP). In MP, an iteration consists of projecting the image onto the dictionary, finding and storing the largest coefficient, and subtracting that element from the image [72]. Iterations continue until a stopping criterion is met. The second method uses Convex Optimization (CO) [73]. The reconstruction problem is formulated as a convex problem in which the variable to be optimized is the sparsity of the coefficient vector (quantified as the L1 norm). Convex optimization attempts to arrive at a solution for the significant coefficients in parallel.

Conceptually, MP is similar to finding coefficients for an orthonormal dictionary. In one iteration of MP, the image is projected onto the dictionary, just as if the dictionary
were orthonormal. However, instead of keeping all the resulting coefficients, only the largest coefficient is stored as part of the solution. Next, that element is subtracted from the image [72]. Iterations continue until a stopping criterion is met, as described below.

Our implementation of the Matching Pursuit (MP) algorithm uses three different stopping criteria. The first stopping criterion limits the number of iterations (i.e. the maximum number of emitters the algorithm will return for a given scene). Simulations indicate the reliability of the algorithms decreases as the emitter density increases. Therefore we do not record results that are likely to be erroneous. Not only is a limit placed on the maximum number of iterations, but the order in which results are subtracted from the scene is also recorded. With this information, we can observe the iterative algorithm as the density increases in an experimental setting.

Another stopping criterion is the minimum estimated number of photons from an emitter. Even when all the emitters in a scene have been accounted for and subtracted, the remainder will still have positive values due to noise (particularly background noise). This could lead to spurious emitters in the reconstruction. To avoid this, MP ends iterations if the photon count of the strongest remaining component is below a threshold. The threshold can be determined beforehand based on the emitters used in the experiment, or decided after the experiment based on the data set. In the latter case, a preliminary threshold must be set to a very low value. Once MP is complete, the threshold is raised and the weakest emitters are excluded from the results.

The final criterion involves placing a limit on the value of pixels in the remainder image. As above, when all actual emitters in a scene are accounted for and subtracted, there will still be a remainder due to noise. Once the maximum pixel value in the remainder image drops below the expected value of background in the data set, the subsequent localizations are most likely the result of noise. Therefore, the iterations are stopped when the maximum of the remainder image drops below a threshold. Typical background levels can be predicted accurately from examining statistics of the whole data set. Furthermore, the threshold is
usually set slightly below the background level (by perhaps 75%) to avoid false negatives. This criterion was found to terminate iterations more frequently than the photon counts criterion, while significantly speeding up the computation time. The reason for the speed increase is because this criterion is evaluated before the remainder image is projected onto the dictionary, whereas the minimum photon number criterion is evaluated after a projection. The speed increase is simply due to a reduced number of computed projections.

Implementations of MP (or other iterative algorithms) in other fields employ a stopping criterion that compares the reconstructed image to the raw data [72]. Iterations stop when the error between the two has reached an acceptable level. Such a stopping criterion is less applicable here because the goal is not to produce an accurate reconstruction of each frame. Rather, the goal is to estimate the locations of emitters. A limit on the error between the reconstruction and the original data would be meaningless in the case of a scene that contains no emitters. In fact, such a stopping criterion would often be at odds with the previous two criteria, and therefore it cannot be seamlessly combined with the other stopping criteria.

3.2.3 Estimation using Convex Optimization

Estimation using CO, on the other hand, attempts to arrive at a solution for the significant coefficients in parallel. Although the estimation method is quite different, the use of the algorithm is similar—the inputs are a window of data and a dictionary, and the output is a list of coefficients that describe the data. The reconstruction problem is formulated as a convex problem in which the variable to be optimized is the sparsity of the coefficient vector (quantified as the L1 norm). This can be phrased mathematically as:

$$\minimize ||x||_1 \text{ subject to } \|Ax - b\|_2 \leq \varepsilon,$$

(3.1)

where $x$ is the set of coefficients, $A$ is the dictionary, and $\varepsilon$ is an error bound related to the total intensity in the image [48]. In this formalism, the image $b$ has been folded into a column vector. In our implementation, we use CVX, a MATLAB package for solving convex
problems [73, 74].

### 3.2.4 Estimation using MP+CO

The Monte Carlo simulations show that while MP is fast, the results are suboptimal. Conversely, the reconstruction obtained with CO is slower, but the returned locations achieve the limit imposed by the fineness of the dictionary. To attain a method that is fast, accurate, and precise we developed a hybrid algorithm that takes advantage of the strengths of both methods. First, MP provides a rough estimate of the number of emitters and their locations. Since the precise location is not needed at this stage, we use a coarse dictionary. Next, CO provides a finer estimation with a refined dictionary. Interestingly, because coarse estimates of the locations are already available from the MP method, we can limit the dictionary to only include elements located close to those estimates. Therefore, we implement a dictionary with the desired fineness while the problem is still computationally tractable. This hybrid algorithm reduces the size of the fine dictionary by nearly two orders of magnitude, enabling CO to be performed in a reasonable time. We refer to this technique as “MP+CO.” At very high densities, MP recalls far fewer sources than CO. In such cases, we have used a CO+CO method, which consists of two or more steps of CO with progressively finer dictionaries. If one were to use MP+CO for very high densities, the initial round of MP might eliminate dictionary elements that are required for the solution. In such cases, the increased computational cost of an additional round of coarse CO is justified.

### 3.3 Monte Carlo Simulations

To demonstrate the performance of the two estimation methods, we present the results of Monte Carlo simulations. Increasing numbers of emitters are placed randomly within a volume, and reconstructions with MP and CO are compared. When making comparisons, we quantify the accuracy of the number of returned emitters and also their locations. To match
our experimental system, the effective pixel size in sample space is 160\textit{nm}, which is due to
the use of a 100x Nikon objective with a Numerical Aperture (NA) of 1.45 and a camera
with 16\textit{\mu m} pixel size. The emission wavelength is 670\textit{nm}. To reflect typical experimental
conditions, each emitter is assumed to produce between 1900 and 2000 detected photons.
Noise is simulated by adding a constant background of 20 photons per pixel, and Poissonian
shot noise is also included. For eight different emitter densities, we simulate three frames of
sample volumes that map to an image 47x47 pixels on a side. We process these frames using
CO and MP and quantify the results in terms of the number of emitters that are correctly
localized and the accuracy to which they are localized in each dimension. A graphical
summary is shown in Fig. 3.3.

3.3.1 Performance Limits of Sparsity-based Methods with the DH-PSF

For MP, the dictionary step size is 10\textit{nm} in the transverse dimensions and 15\textit{nm} in
deepth, yielding 1.27 million dictionary elements. Even with such a large dictionary, one
MP solution on an 11x11 window can be completed in tenths of a second on a desktop
computer. If the reconstruction returns the correct elements, there will still be localization
errors due to the quantization of the solution space. For this dictionary, the quantization
error will be 2.9\textit{nm} in each transverse dimension and 4.3\textit{nm} axially. The quantization error
bounds indicate the error if the correct dictionary element is chosen every time. In the
simulations, MP does not perform to the limit of the dictionary. The source of the non-
deal performance of MP is likely due to the so-called “greedy” nature of the algorithm;
namely, with each iteration, the largest possible portion of the image is subtracted. This
drawback is easily offset by using a finer dictionary. The density of correctly localized
emitters (herein named the “recovered density”) can be as high as 1 emitter/\mu m^2, which is
twice the maximum recalled density of 3D-DAOSTORM with an astigmatic PSF [52]. More
importantly, this recovered molecule density is more than seven times higher than existing
3D methods, including DH-PSF, astigmatic, and bi-plane techniques [52].
Figure 3.3: **Performance of different super-resolution methods as a function of the molecule density evaluated via Monte Carlo simulations.** For each density, three simulated frames with a size of $55\mu m^2$ were generated. Then, the results from Matching Pursuit (MP) and Convex Optimization (CO) are quantified and compared to 3D-DAOSTORM (3DDS). (a) shows the number of correctly localized emitters. This plot shows that CO can recover the highest molecule density. The localization error is shown in (b-d) for transverse (x,y) and axial (z) localization, respectively. These plots demonstrate it is possible to maintain good localization precision, even at very high densities. For MP, the dictionary had $10nm$ transverse step sizes and $15nm$ axial steps. The dictionary for CO had $80nm$ and $120nm$ steps in the transverse and axial dimensions, respectively.

Due to current computational limitations, the dictionary for CO cannot be as large because it is a much more computationally intensive algorithm. Thus, we select a coarser dictionary with transverse steps of $80nm$ and axial steps of $120nm$ (2904 elements). The effect of quantization error for this dictionary is $23nm$ in the transverse dimensions and $35nm$ axially. The simulations in Fig. 3.3 indicate CO performs very close to the limit of the dictionary. Even with such a significantly smaller dictionary, CO still requires an order of magnitude more computation time than MP with a finer dictionary (see Section 3.3.4 for more details regarding the calculation time of the algorithms). However, CO performs
better than MP in terms of recoverable density. The enhancement enables the reconstruction of frames with an emitter density of more than 1.5 emitters/$\mu m^2$, which is a three-fold improvement in recoverable density over 3D-DAOSTORM [52], and more than an order of magnitude improvement over established 3D localization schemes (DH-PSF, astigmatic, and bi-plane).

The simulated images in Fig. 3.4 exemplify the degree to which molecule density is increased to accelerate 3D localization microscopy. Previous localization methods required completely isolated single molecule images, with even nearby emitters causing errors. By enabling the localization algorithm to reconstruct an image with a ten-fold increase in the number of emitters per frame, single molecule microscopy can be accelerated proportionally.

Figure 3.4: **Examples of sparse reconstructions for different density levels.** In both simulated images, we randomly placed emitters in the simulation space, and we applied our localization method. The resulting locations are marked as x’s. In (a), the recovered density is 0.12 emitters/$\mu m^2$, which is approximately the limit of existing localization schemes for the DH-PSF. The recovered density in (b) is an order of magnitude higher. This increased recoverable density means 3D super-resolution imaging experiments can now be performed in 1/10th the time. In these images, system parameters were matched to experimental conditions, as discussed in this chapter.
3.3.2 Demonstration with an Astigmatic PSF

The work presented up to this point uses the DH-PSF, but there are other methods to convey depth information. We performed simulations to confirm that our methods will work for other PSFs. An example using an astigmatic PSF is shown in Fig. 3.5. The top row (a-c) shows a successful simulation using MP. In this case, there are three emitters in the scene, with some overlap for the two defocused emitters.

All emitters are localized successfully, although there is significant error for the dimmest emitter. The second row of Fig. 3.5 (d-f) shows a common problem with MP when the astigmatic PSF is used instead of the DH-PSF. Two bright, in-focus emitters are mistakenly combined into one defocused emitter. The mathematical reason is obvious: since there is a defocused PSF in the dictionary with significant overlap with the two sources, this element will have a much higher coefficient than the two correct PSFs. This is the primary cause of incorrect localizations of overlapping astigmatic PSFs. This issue does not arise with the DH-PSF because the concentration of intensity does not change as quickly in the axial direction as in the astigmatic PSF. When two DH-PSFs are too close to be resolved, the returned localization is between the two true locations. With the astigmatic PSF, the unresolved PSFs can be mistakenly identified as a single source in a very different plane. In any case, there are solutions to avoid this problem in MP for the astigmatic PSF. If a returned coefficient is too large for what can be expected from the emitters in the sample, this result can be excluded from the series, and the iteration can be repeated. Nevertheless, CO was used successfully to localize the emitters on the same simulated data. The results are shown in the bottom row of Fig. 3.5 (g-i). The problem is avoided with CO because the stipulation that the reconstructed image matches the original data is enforced more strongly.

As further evidence that the methods developed in this chapter can be used with other PSFs, we performed Monte Carlo simulations with the astigmatic PSF. Results are presented in Fig. 3.6. The parameters are identical to the simulations in Section 3.3. In fact, these
Figure 3.5: **Demonstration of 3D super-resolution and super-localization with an Astigmatic PSF.** Simulations with an astigmatic PSF. The top row shows a successful localization of overlapping emitters using MP. The simulated frame is shown in a), the true and returned locations are in b), and the reconstructed image is in c). The second row demonstrates a common problem encountered in simulations with closely-spaced astigmatic PSFs. Here, two in-focus PSFs are incorrectly grouped into one defocused (elongated) PSF. However, this problem was not encountered when using CO, as seen in the localizations in h) and the reconstructed image in i). In all images, scale bars are 1 µm (scale bars omitted in d-i to avoid interfering with the image, but the scaling is the same as in a,c).

Plots suggest the astigmatic PSF allows for higher densities. This is due to the fact that the astigmatic PSF is more compact than the DH-PSF, so overlap does not become a problem until higher densities. The advantage of the DH-PSF is the extended axial range and better localization precision, particularly in z. The extended axial range is not reflected in these plots, since the simulations were limited to a range of +/- 500 nm from focus. However, the
axial precision is slightly better for the DH-PSF, which is justified by examining the CRLB analysis in [36]. Furthermore, the use of a hybrid method would permit a finer dictionary, and could improve the localization of the DH-PSF.

Figure 3.6: **Monte Carlo simulations with astigmatic PSF.** As in Fig. 3.3, a) shows the density of the emitters that were correctly localized by MP and CO. The localization precision, as measured by the standard deviation of the errors, are shown in (b-d) for x, y, and z respectively. At each density, three simulated frames with an area of 55 µm² each were generated. The random locations for the emitters are the same as those used in the simulations for the DH-PSF. The dictionary step sizes are also identical; MP used transverse and axial step sizes of 10 nm and 15 nm, and CO used 80 nm and 120 nm steps.

In conclusion, even though most examples shown in this chapter use the DH-PSF, the methods discussed in this Dissertation are equally applicable to other appropriate 3D PSFs.

### 3.3.3 Comparison of PSFs with Depth

It has been shown that for isolated emitters, the DH-PSF allows for longer axial range localization than the astigmatic PSF [35]. Here we show that a similar behavior is revealed for dense arrays of emitters. For this purpose we performed a simulation with an axial range of 2 µm, including five 7x7 µm frames of high emitter density (2 emitters/µm²). Each emitter is given a random intensity between 2900 and 3100 photons. The background noise level is 10 photons/pixel, and shot noise is also included. Since the astigmatic PSF increases in
size with defocus, the dictionary window size is 16x16 pixels (we typically use 11x11 pixel windows). All dictionaries have 80nm transverse steps and 100nm axial steps, which results in 21504 elements.

These simulations include two versions of the DH-PSF. One is the numerically optimized version [34], and the other is the analytic DH-PSF (first described in [36]). To differentiate, the numerical version is referred to as DHPSF-N and the analytic is called DHPSF-A. Generally, most statements in this chapter refer to DHPSF-A, and all other simulations outside of this section use the DHPSF-A.

Figure 3.7 summarizes the results of these Monte Carlo simulations. In addition to considering three different PSFs, the results are also sectioned into three different ranges of defocus. Emitters are classified as “in focus” if they are within $0.33 \mu m$ from focus. Those that lie between $0.33 \mu m$ and $0.67 \mu m$ are classified as “mid focus.” Beyond $0.67 \mu m$, the emitters are called “far focus.” This concept is demonstrated graphically in Fig. 3.7 a). The remaining plots in the figure use this classification for the abscissa. The plot in Fig. 3.7 b) shows the recall fraction for the different PSFs and different focal regions, and plots c)-f) show the localization errors. For thin samples where the emitters are within $0.33 \mu m$ of the focus, the astigmatic PSF performs best. However, for emitters far from the focus, the astigmatic has the worst performance in recall fraction and 3D localization error. The behavior of the astigmatic PSF readily explains this trend; near focus, the PSF is compact, which enables good performance for resolving nearby emitters in high density scenes. Furthermore, the compact PSF provides an advantage in terms of signal to noise. As the distance from focus increases, the astigmatic PSF spreads out quickly compared to the DH-PSFs. The DHPSF-A is also quite compact near focus, but spreads out more slowly than the astigmatic. This tendency is reflected in the performance in terms of recalled fraction and localization error. The DHPSF-N is larger, which explains the lower recall fraction. However, this PSF maintains integrity throughout the full $2 \mu m$ range, and thus experiences comparatively little degradation far from focus.
Figure 3.7: A comparison of different PSFs through an extended axial range. The classification scheme of axial sections is demonstrated in a). The performance of recall fraction as a function of defocus is shown in b) for three different PSFs. The localization error in x, y, and z is plotted in c)-e), and f) shows the 3D localization error (calculate as the square root of the sum of the squares in each dimension).

Interpretation of these results reveals a general trade-off between performance near focus and performance over a long axial range. Intuitively, this can be understood by pondering the inverse relationship between the spot size and divergence angle of a focused Gaussian beam. A more compact PSF will perform better near focus, but will diverge quickly and suffer correspondingly far from focus. Conversely, a less compact PSF will not perform as well near focus, but will provide more consistent performance over a longer axial range. Fur-
thermore, the parameters that define the DHPSF-A (as presented in [36]) allow for a tuning of the PSF properties to fit specific experimental needs.

### 3.3.4 Calculation Time

Here, we provide information on the calculation time required to complete the localization algorithms. All computational work was performed on a desktop computer with an Intel i7-860 Central Processing Unit, which has four cores running at 2.8GHz. The machine has 16GB of random-access memory. Algorithms were written using MATLAB Version 8.2.0.701 (R2013b) in a 64-bit Linux operating system. This CPU has a rated performance of 82,300 Million Instructions Per Second (MIPS) according to the Dhrystone performance benchmark. We use this performance benchmark to convert between calculation time and number of instructions. Anyone wishing to replicate these methods can estimate the calculation time by dividing the number of instructions by their CPU Dhrystone MIPS rating.

The time required to complete the localization of one 11x11 pixel window for MP and CO as a function of emitter density is plotted in Fig. 3.8. For these simulations, a coarse dictionary with 5324 elements was used. For each point in Fig. 3.8, 50 frames were processed. Each frame was 56 pixels on a side, resulting in a 6x6 sub-windows per frame (with a two-pixel overlap between sub-windows). This means each point and its corresponding standard deviation error bars reflect a total of 1,800 sub-windows.

There are two main conclusions that can be drawn from these plots. First, as previously stated, CO is significantly more computationally intensive; it requires more than three orders of magnitude longer to address the same 3D estimation problem. Clearly, CO should only be used when the density is sufficiently high to warrant the long calculation time. From Fig. 3.3, the threshold at which CO begins to outperform MP is between 0.5 and 1 emitters/µm².

Secondly, the change in calculation time as density increases is different for the two methods. As one would expect, calculation time for MP increases approximately linearly with emitter density. More emitters means more iterations of MP, and thus more multiplications of
Figure 3.8: **Calculation time as a function of emitter density.** These plots show the calculation time and number of instructions required to process one 11x11 pixel window of data. Plot a) shows the time required for MP, and plot b) shows the time required for CO. The right-hand y-axis of both plots shows the number of iterations these times correspond to for our processor.

the data and the dictionary. CO calculation time, on the other hand, does not change significantly over the useful range of the method (0.5 to 2 emitters/µm²). In CO, the algorithm is optimizing the sparsity of the vector containing the coefficients that describe the data. The initial guess at the solution is typically not sparse, but contains many non-zero coefficients. Depending on the size of the dictionary, the initial guess may contain many thousands of non-zero components. As iterations progress, the number of non-zero components is reduced to a smaller and smaller number, eventually reaching a solution in which only a few components remain. This explains why the time required does not differ over the range of emitter densities. If the final solution contains only between one and six non-zero components, the number of iterations to reach this solution from an initial guess of many thousands of non-zero components does not result in a large change in calculation time.

### 3.4 Experimental Results

#### 3.4.1 SPINDLE Experimental Description

To convert a standard microscope to a DH-PSF system, the image plane is relayed using a “4f” system (two lenses separated by the sum of their focal lengths). The PSF is
modified by placing a phase mask in the central plane of the relay system. A sketch of the system used to collect the experimental data is shown in Fig. 3.9. The sample is mounted on a piezo nano-positioner. The deactivation laser is a Coherent Cube diode laser (641\text{nm}) and the activation laser is a Coherent argon ion laser (488\text{nm}), which are synchronized with the camera. The imaging system is comprised of a 1.45NA 100x Nikon objective, a 200\text{mm} tube lens, and 100\text{mm} lenses for the relay system. All lens diameters in the imaging system are 50\text{mm} in diameter. The camera is an Andor iXon+ EMCCD with 16\mu m pixels. The samples were prepared following the protocols described in [36]. The emitters in the experiments provide 500 to several thousand photons detected per frame. The median background photons per pixel per frame is 30 for the entire dataset. In the dense frames (as in Fig. 3.10), the background was 50 photons.

![Experimental System Diagram](image)

**Figure 3.9: Experimental System Diagram.** The optical set-up for the SPINDLE microscope is shown here. The upper half of the set-up is the illumination section, with the appropriate optics for combining the activation and deactivation lasers. The imaging path is the bottom channel. The dashed line is the intermediate image plane (a system with a standard PSF would place the camera there). To the right of this image plane are the optics for the 4f relay system. Bandpass filters are labeled with BP.
3.4.2 Anecdotal Results

Two examples of experimental scenes (raw data) of dense molecule clusters are shown in Fig. 3.10. These images are acquired using a SPINDLE system [36] incorporating a DH-PSF and stochastic optical reconstruction using photoswitchable dyes [21]. In the scene in Fig. 3.10 (a), there are two bright lobes, with a third dim lobe nearby. There are likely two emitters; one bright emitter, and a dim emitter nearby with one lobe coinciding with a lobe of the bright emitter. The hybrid method is able to identify the individual emitters despite the overlap of the lobes. The scene in Fig. 3.10 (d) is even more complex. MP+CO is able to resolve and localize three emitters in this scene. Such a scene would be rejected from typical localization algorithms, and none of the emitters would contribute to the final image.

Figure 3.10: Experimental demonstration of 3D super-resolution and super-localization from overlapping single-molecule images. (a) and (d) Two examples of raw data of overlapping molecule images using a DH-PSF system. (b) and (e) show the estimated locations of the molecules using the MP and hybrid methods. The regions used for the refined dictionary are marked with blue cubic boxes. The images in (c) and (f) show the reconstructed image using MP+CO. In all images, scale bars are 1µm.
3.4.3 Large Scale Reconstruction

An example application in a large-scale super-resolution image is shown in part (b) of Fig. 3.11. For comparison, a standard fluorescence image is shown in part (a) of the same figure. The sample is composed of PtK1 cells (Rat Kangaroo Epithelial cells) in which tubulin is labeled with Alexa-647 and Alexa-488 dyes. The reconstruction image was compiled from more than 30,000 frames, and the image clearly demonstrates 3D super-resolution capabilities. In the standard fluorescence image of this scene, many of the individual microtubules either cannot be resolved, or are out of focus due to the limited depth of focus of the standard PSF of high-NA objectives.

The super-resolution image in Fig. 3.11 (b) was generated by assigning a Gaussian spot to each localized emitter. The width of the Gaussian is inversely related to the square root of the number of photons received by the emitter (a higher photon count means the Cramer-Rao bound is lower, i.e. the localization precision is better). The color of the spot is determined by the depth of the emitter.

3.5 Quantitative 3D Measurement of Sub-cellular Structure

3.5.1 Measurement of Microtubule Radius

Although simulations already indicate these sparsity-based methods can allow high label densities without sacrificing the ability to achieve super-resolution, we also evaluated the performance on experimental data. Figure 3.12 (a) shows a small region of the super-resolution image from Fig. 3.11. As opposed to the prior image, here the localizations are visualized in a scatter plot, although depth is indicated with color as before. The set of 131 locations was fit to a straight line in 3D space (see Section 3.5.3 for an analysis of the 3D sampling of this structure). These points were then converted to a 2D space which defines the transverse and axial distance from the fit line to each point. Here, “axial” is referring to the optical axis of the microscope. This view is shown in Fig. 3.12 (b). Equivalently, one
Figure 3.11: **Large-scale experimental implementation.** A standard fluorescence image is shown in (a). The 3D super-resolution image (b) of labeled tubulin in PtK1 cells demonstrates that the method can be applied to localization-based super-resolution imaging with a wide field of view. The small box on the right side of (b) indicates the region that is used for subsequent detailed analysis. Scale bar: 1 μm.

We can think of this as a view of the 3D cloud of points from a position along the microtubule. The ellipse shows the standard deviation of the points along each axis. The dimensions of this ellipse are 82 nm axially (z) and 32 nm in the transverse dimension (x,y). These points are then converted to cylindrical coordinates and plotted in Fig. 3.12 (c), revealing a lack of molecules for low radial distances. The ellipse from (b) is also converted to cylindrical coordinates and shown in (c). Next, we calculate a histogram of the radial distance from the fit line, which is shown in (d). From this histogram, we observe that the object we are reconstructing is not simply a line, but in fact a cylinder. The method is not only able to generate large images with enhanced resolution as in Fig. 3.11, but is in fact producing quantitative measurements of 3D properties of the microtubules. Since the precision differs
Figure 3.12: **Measurement of the 3D cylindrical structure of an antibody labeled microtubule.** A small region of the data containing a straight segment of a microtubule is shown in (a). In the context of the large image in Fig. 3.11, this region is indicated with a small white box on the right side of the image. These points are fit to a line in 3D space, and the distance from each point to the fit line is shown in (b); this can also be thought of as a view of the microtubule from the perspective of the microtubule axis. The same plot is converted to cylindrical coordinates and displayed in (c). Plots (d)-(f) all show histograms of the radial distance of the points to the fit line; (d) shows all points, and (e) and (f) show the points classified as transverse and axial points, respectively. From these histograms, it is apparent that we are observing emitters that are attached to a cylindrical object. Simulations in the following section verify that the distributions observed here are consistent with a microtubule with a 35 nm radius.

Significantly in the axial dimension, we classify the location of the points as either “axial” or “transverse”, as indicated by the color and shape of the points in (b) and (c). The anisotropy in localization precision is responsible for more points being classified as “axial” rather than “transverse,” i.e. even with a uniform distribution of labels, the distribution of localizations will be skewed along the axial direction. The simulations presented in the following section exhibit the same behavior. We calculate the separate histograms of the radial distance for the two classifications of points and plot them in (e) and (f). From these
histograms, we measure the median radial distance to be 49\textit{nm} and 75\textit{nm} in the transverse and axial dimensions, respectively.

Prior reports have shown that antibody labeled microtubules have a radius of about 30\textit{nm} \cite{75, 76}. We performed an analysis taking into consideration the anisotropic precision of 3D localization leading to an estimate of the radius at 35\textit{nm}. Our analysis based on the results of Fig. 3.12 was obtained with a median of 614 detected photons per molecule. The experimental photon count is lower than the values used in simulations, resulting in lower localization precision \cite{24}. On the other hand, lower precision enables the use of a coarser dictionary. The predicted experimental localization precisions for that intensity level are 30\textit{nm} and 66\textit{nm} in the transverse and axial dimensions, respectively \cite{36}. Such anisotropy causes the circular cross-section of the microtubule to appear elliptical, as mentioned previously. Subsequently, we estimate the radius of the antibody labeled microtubule based on the known localization precision of 131 emitters fitted to the observed elliptical cylinder, leading to a precision surpassing that of an individual localization. The estimation result for the radius, including the antibody labeling structure, is 35\textit{nm} \pm 12\textit{nm}. The error is given as one standard deviation. A slightly larger radius could be attributed to a minor bending of the microtubule or simply to the low photon count of our measurements. It should be noted that a cylindrical shape of microtubules has been previously observed \cite{77}, albeit in 2D and with emitters that yield more than 100 times more photons than in our experiment.

3.5.2 Simulation of Point Sources on a Cylinder

In the previous section, we presented an analysis of a short section of a microtubule. We demonstrated that the emitters were distributed on an elliptic cylinder, then claimed those results are consistent with a distribution that is actually a circular cylinder that was localized in a pointillistic fashion with anisotropic precision. To support this claim, we present a simulation in which 1000 emitters are placed randomly on a circular cylinder with a radius of 35\textit{nm}. Then, each location is perturbed with a localization error with a standard deviation of
30nm in the transverse dimension and 66nm in the axial dimension. These values correspond to the experimentally measured precision for sources providing 614 photons to our imaging system [36]. Figure 3.13 represents the molecule location in a presentation analogous to that used in Fig. 3.12. As the histograms indicate, there is a notable lack of emitters localized at low radial distances from the axis of the simulated microtubule. As in Section 3.5.1, the locations are classified into transverse and axial locations due to the difference in precision in those dimensions. From the histograms, we observe a median radial distance of 50nm for transverse localizations and 74nm for axial localizations. Similarly, the observed values from the experimental data were 49nm and 75nm in the transverse and axial dimensions, respectively. Regardless, these measurements indicate that the method is sensitive to the 3D structure of the microtubule, not only helping resolve nearby microtubules, but also revealing structural details smaller than the microtubule radius of 30nm.

3.5.3 Sampling Considerations

In this section, we present some basic calculations regarding the density of emitters along the biological structures analyzed in Section 3.5.1. Specifically, we address the density of emitters analyzed in Fig. 3.12. In that case, we found 131 emitters along a 1µm length of microtubule. This corresponds to one emitter every 7.6nm along the one-dimensional length of the microtubule. For the number of photons detected per emitter in our experiments, the localization precision is more than twice this value. Therefore, the sampling density is sufficiently high to reconstruct the microtubule as a line.

However, we demonstrated that the level of resolution is such that one can observe that the emitters are not distributed along a one-dimensional line, but rather a cylindrical structure. If we assume this cylinder to have a length of 1µm and a radius of 30nm, the two-dimensional labeling density for the 131 emitters is 695 emitters/µm². This is equivalent to allowing the emitters to be spaced evenly in a square grid pattern on the surface with 38nm between neighboring emitters. For comparison, an emitter providing 500 photons
Figure 3.13: Simulated results of localization microscopy on a circular cylinder with a radius of 35nm. Each of the 1000 emitters are placed on the surface of a microtubule, then given an axial and transverse location error corresponding to the predicted precision of a DH-PSF. The resulting locations are shown in a) along with an ellipse showing the standard deviation of the locations in the transverse and axial dimensions. The same points are shown in b), but in cylindrical coordinates. Note the lack of points at low radial values. This observation is very clear in the histogram of radial distances of all points, as shown in c). This histogram is split into points classified as transverse and axial points, and the resulting histograms are shown in d) and e).

has a CRLB of 28nm and 70nm in the transverse and axial dimensions, respectively. One could interpret this as meaning that an emitter providing 500 photons has a two-dimensional uncertainty area of $1960nm^2$ for surfaces that lie along the optical axis (the x-z or y-z planes); the inverse of this value is $510$ emitters/µm$^2$. This suggests that a labeling density of 2040 emitters/µm$^2$ would be desirable to satisfy Nyquist sampling criteria of the 3D structure under study for the case where the surface lies along the optical axis. Similarly, for surfaces in the x-y plane, the two-dimensional uncertainty area is $(28nm)^2 = 784nm^2$, resulting in a desired labeling density of $5102$ emitters/µm$^2$ to satisfy Nyquist. However, the assumption
that the object under study is cylindrical and has a constant radius relaxes this requirement significantly.

3.6 Discussion and Conclusion

The origin of the super-resolution capability is interesting to ponder, given that the raw images are limited by diffraction and deconvolution techniques provide only limited success [78]. First, an engineered 3D PSF is essential to retrieve 3D information, as is a 3D dictionary. Second, the fundamental assumption of sparsity, i.e. only up to a handful of emitters are located within the PSF area, provides the required prior knowledge to achieve effective resolution and localization. Hence, super-resolution and super-localization are ultimately enabled by the combination of 3D optical techniques and prior knowledge. Furthermore, these two concepts can be linked in that the increased structure of the DH-PSF contributes to the solution of the localization problem. While the task of correctly pairing lobes of overlapping DH-PSFs might be thought of as an inherent disadvantage of the PSF, the lobes have a particular shape and separation that can help discern the underlying emitter locations.

An important difference between the methods in this Dissertation and other sparse reconstruction schemes [79] is that our goal is not to generate a reconstruction of each image. Although a reconstruction image is obtained, the desired information is actually in the indices of the large coefficients in the sparse reconstruction. Each dictionary element has a physical meaning: the presence of a large coefficient in the solution means there is an emitter at the corresponding location.

In conclusion, the method presented here has the capability to resolve dense clusters of molecules (or other emitters) from a single image in three dimensions while maintaining the high 3D localization precision. Therefore, higher labeling density and significant decreases in data collection time for 3D super-resolution microscopy experiments are now possible.
Because the technique enables super-resolution imaging with far fewer image frames, hence reducing the data required to reconstruct a nanoscale resolution image, the method can be classified as a compressive imaging technique.
Chapter 4

Enhancing Localization Microscopy for Multi-color Thick Samples

The initial demonstrations of super-resolution were performed on custom systems and involved teams with specialization in biochemistry and biology, optical system design, and image processing. The breadth of knowledge and experience required for such experiments has slowed the adoption of super-resolution microscopy techniques by a broad range of users. This situation is changing as the field matures. More probes are available off-the-shelf, and sample preparation methods are well documented. Commercial systems have been made available in recent years. However, the applications of super-resolution imaging have yet to reach their full potential. The work presented in this chapter aims at making super-resolution techniques more accessible by presenting experimental and computational techniques to address problematic issues arising from the application of super-resolution imaging to new problems.

The data presented in this chapter is the result of a collaboration involving the investigation of viral reproduction of the Murine Polyomavirus within the nucleus of a mouse embryo fibroblast cell. One of the challenges of this project is the thickness of the cell and the density of the proteins of interest therein. Furthermore, the multiple proteins involved in viral reproduction and virion packaging necessitates multi-color imaging. SMLM is promis-
ing for viral studies because it can bridge the gap in resolution between electron microscopy and confocal, but the small size of viruses and viral products creates a challenge even for SMLM [80]. Super-resolution has been demonstrated within the nucleus of cells in non-viral applications [81, 82, 83], but the targets under investigation were not present in the high densities typical for virally-produced proteins.

The major problem presented by a thick sample is excessive background due to out-of-focus fluorophores, as shown schematically in Fig. 4.1. In one early demonstration of localization microscopy, the motion of the Kinesin motor protein was determined to be a “hand-over-hand” mechanism [66]. In this experiment, the protein was observed while interacting with microtubules immobilized on the surface of a coverslip, and TIRF excitation ensured a minimum of out-of-focus fluorescence. Furthermore, the density of targets was very low and the experiments did not seek to attain superresolution. The initial PALM paper restricted regions of interest to thin cryosections or cell surfaces where TIRF excitation was possible [20]. Similarly, the first FPALM paper imaged photoactivatable green fluorescent protein on coverslips and crystal surfaces [22]. The earliest STORM paper examined DNA immobilized on a coverslip and also employed TIRF excitation [21]. Solutions to the problem of thick samples include light sheet illumination [84, 85], Bessel beam plane illumination [86, 87], two-photon absorption [4, 88], and temporal focusing [89, 90]. Although effective, these methods require more complex and costly optical systems and do not promote easy access to a wide range of users. In the work presented here, screening of the available photoswitchable dyes led to a choice of dye that performed well despite the sample thickness.

An additional problem of thick samples involves drift correction. Typical approaches use fiducial markers to track sample motion over time. The fiducials are typically fluorescent beads, which are very bright and thus provide excellent localization precision. Drift can be corrected after localization, or it can be counteracted in real time [91, 92]. To avoid drowning out the relatively weak signal of single molecules, the fiducials are deposited sparsely to avoid overlapping with a region where localizations are required. However, when imaging a plane
Figure 4.1: **Difficulties with localization in thick samples.** The problem of excessive background due to out-of-focus fluorophores in thick samples reduces the localization precision in super-resolution microscopy. Furthermore, drift correction using fiducials is not applicable when the coverslip/sample interface is far from the focal plane. Hence, fiducial-free drift correction methods are required.

several microns above the coverslip, fiducials are out of focus, as seen in Fig. 4.1. Other drift correction methods use image correlations to estimate drift [93, 94]. The drift correction method I propose exploits the repeated activations of dyes to estimate drift.

There are numerous multi-color SMLM demonstrations [31, 76, 95, 96, 97, 98], albeit in thin samples. For example, the two sample types in [76] are DNA immobilized on a surface and microtubules. Although microtubules can get quite dense in the middle of a cell, most SMLM focus on regions at the edge of a cell where the sample is thin. The structures in [95] are microtubules as well. There are also multicolor 3D SMLM demonstrations [96, 97, 98]. Although the optical system in Ref. [96] has a depth of field of $\sim 2\mu m$, the objects are bacteria with a thickness of $\sim 600nm$. The 3D methods in Refs. [97, 98] limit the sample thickness to less than $\sim 1\mu m$. A thorough investigation of photoswitchable dyes for multi-color imaging is given in [31]. The main objects imaged are typically microtubules, although
several other structures have been used which are more dense than microtubules at a cell’s edge. The multi-color results shown here are within cell nuclei with thicknesses ranging from 7-10\(\mu m\).

The first section of this chapter describes the optical system for multi-color SMLM. Next, the topic of fluorophore selection is addressed in order to find a pair of dyes that exhibit favorable characteristics for localization microscopy in a thick sample. The choices in fluorophore selection were guided by the processing algorithms described in Section 4.3. The two-color SMLM data from the virus-infected nuclei demanded a fiducial-free drift correction method, which is outlined in the same section. Subsequently, the rendered super-resolution images of infected cells are shown. Lastly, observations regarding multi-color 3D SMLM suggest a path forward for outfitting microscopes with 2-color 3D.

4.1 Optical System for Two-Color Localization Microscopy

A microscope for performing STORM has several distinctions from a standard fluorescent microscope. This section describes the optical system necessary for performing STORM experiments. First, the basic system layout is presented. Then, additional details are provided regarding the requirements for excitation and reactivation lasers. For multi-color STORM, special attention must be given to spectral filtering of the channels (see appendix F). The optical system presented here is based on a Nikon commercial system, although modifications were made to adapt the system to our specific experimental requirements.

The basic optical system for a STORM microscope is separated into the excitation path and the imaging path. Excitation occurs with epi-fluorescence, meaning the illumination light is focused by the same objective that is used for collecting emission light. For thin samples that are close to the coverslip/sample interface, background can be minimized by using Total Internal Reflection Fluorescence (TIRF). In the TIRF modality, the excitation
light does not propagate through the depth of the sample but instead only excites a thin layer at the interface. For thicker samples, TIRF is not an option. However, the background can be reduced to a lesser degree by using oblique illumination. A TIRF illuminator, as shown in Fig. 4.2, provides control over lateral displacement of the beam before the objective. Thus, one can control the angle of propagation of the excitation light within the sample. The illumination system contains multiple lasers for excitation/deactivation and reactivation of a variety of fluorophores. Additionally, each laser is equipped with an acousto-optic tunable filter for shuttering.

The emission light is separated from the excitation light with a dichroic mirror. To avoid switching the dichroic during acquisition, the dichroic has a quad-band reflection profile which reflects the lasers but transmits emission light from most fluorescent probes (see Fig. F.2 on page 128). The transmitted light is spectrally filtered again with a bandpass to remove any remaining light from the comparably high-intensity excitation light. The dichroic and filters are placed in infinity space to avoid introducing spherical aberrations that arise from placing flat elements in a converging beam.

The imaging path is comprised of the coverslip, the objective, the tube lens, and the camera. Unless otherwise noted, the experiments presented here use a 100x 1.49NA oil-immersion objective from Nikon. As per the design, this objective is paired with a tube lens with a 200 mm focal length. The camera is an Andor iXon Ultra 897 Electron Multiplying Charge-Coupled Device (EMCCD) with 16 µm pixels. This combination of magnification and camera pixel size results in an effective pixel size of 160 nm in sample space. For comparison, the PSF of a point source that emits at 670 nm has an Airy disc diameter of 549 nm and a full-width half-max of 231 nm. According to Nyquist criterion, the PSF is slightly oversampled; there are 3.5 pixels across the diameter of the Airy disc. For some experiments, I use a Hamamatsu Orca Flash 4.0 CMOS (Complementary MetalOxideSemiconductor) camera; the 6.5 µm pixels necessitate an objective with lower magnification. The Nikon 60x 1.49NA objective, when used in combination with the CMOS, results in an effective pixel size of
The microscope base is situated on a vibration-isolating table. The sample sits on a piezo-activated sample stage which allows fine control of the 3D position of the sample relative to the objective. The microscope base also contains turrets for selecting the proper objective and dichroic cube. The entire system is built around a commercial product from Nikon called the “N-STORM.”

Figure 4.2: **Dual-camera STORM configuration.** Two-color STORM with Atto 565 and Alexa 647 requires careful filtering of the two channels to avoid excessive background. In this layout, an second dichroic splits the two emission colors onto separate cameras.

### 4.2 Fluorophore Selection

The experimental aspect of performing a STORM experiment can be further subdivided into two parts: sample preparation and data acquisition (which includes the optical
apparatus). As described in Chapter 1, the key assumption in a SMLM experiment arises from the ability to switch fluorophores in time between an active state and a dark state. Figure 4.3 shows a possible switching mechanism that explains the presence of the active and dark states. On the left is a structural model of an active cyanine dye, “Cy5,” before illumination. When the dye is excited in the presence of the thiol $\beta$-mercaptoethanol ($\beta$ME), spectral and mass spectrometry measurements suggest the presence of the species shown on the right of Fig. 4.3. The thiol has attached to the “bridge” of the dye, resulting in a quenching of the emission light. Illumination with a UV reactivation laser induces the dye molecule to release the thiol, thus reverting back to the active state on the left [1].

Figure 4.3: **Dye switching mechanism.** The structural model of a Cy5 dye in an active state is shown on the left. In the presence of the thiol $\beta$-mercaptoethanol, illumination causes the dye to convert to the dark state shown at right. Illumination with UV light (e.g. a small dose from a 405nm laser) reactivates the molecule back to the active state. (Figure adapted from [1].)

An understanding of the switching mechanism informs the sample preparation. The first aspect of sample preparation is proper labeling using a photswitchable dye. The typical labeling method is a two-step process: first, a primary antibody designed to specifically attach to the target object is introduced to the cell. Next, a secondary antibody is introduced. The secondary antibody recognizes the primary antibody and attaches. The other end of the secondary antibody has been conjugated to one or several dye molecules. The second aspect of sample preparation is the use of the quenching buffer containing the thiol that switches the dyes to a dark state. There are many dyes available that exhibit photoswitching capabilities. Each dye differs in wavelength, intensity, quantum yield, photostability, and duty cycle (ratio of active and dark molecules) [31]. The selection of the quenching agent also affects
these properties. The composition of the buffer containing the quencher can also influence performance [99]. For example, the addition of an oxygen scavenging system such as glucose oxidase with catalase can improve photostability and reduce undesirable photobleaching [31].

Proper dye selection can greatly affect the quality of STORM images and measurements. The best dye(s) can also vary depending on the requirements of an experiment. Important factors to consider are: sample thickness, density of the target, background levels, and the need for 3D information. Some experiments also require knowledge about the relative position of multiple targets. Fortunately, the wide range of dye choices make such multi-color STORM experiments possible [95, 76, 77, 100]. In the discussion that follows, I present measurements aimed at determining the best dye pair for two-color STORM experiments in the nucleus of a virus-infected cell with a high density of targets. Although the application I am presenting is specific, the choice of fluorophores is applicable to other multi-color experiments in thick, dense samples.¹

To observe two different objects of interest, one must be able to differentiate the two fluorophores used for labeling. The most common way to differentiate the labels is to use spectrally distinct fluorophores. Table 4.1 classifies STORM dyes into four categories based on the spectral ranges of the excitation and emission [31]. The two most common dyes in STORM are Alexa 647 and Cy5. These dyes are favorable because they outperform other dyes in overall performance, especially due to their high photon yield, excellent photostability, and low duty cycle (ensures sparsity). These two dyes also respond well to reactivation. We used Alexa 647 in the experiments presented here. The problem is to find a dye that can perform well alongside one of these red-absorbing dyes.

A second option for differentiating multiple targets is to use dyes that emit at the same wavelength, but can be selectively activated. This behavior can be achieved using “activator-reporter pairs” [76]. Rather than using a single emitter on the secondary antibody,

¹ The work in Sections 4.2 and 4.4 was performed in collaboration with Katie Heiser from the Molecular, Cellular, and Developmental Biology Department at the University of Colorado Boulder.
two species of emitters are conjugated to the secondary antibody. The longer-wavelength fluorophore is the “reporter” dye, meaning this is the color that is actually localized. The “activator” dye serves to absorb reactivation pulses and induce the reporter to re-enter the active state from the dark state. There are numerous options for pairs; by selecting the same emitter as the reporter but different activator dyes, one can selectively activate distinct populations of fluorophores. However, this method suffers from the possibility of spontaneous reactivation, leading to confusion of targets.

Table 4.1: **STORM dye spectral ranges.** This table shows the excitation and emission ranges for dyes for use in STochastic Optical Reconstruction Microscopy.

<table>
<thead>
<tr>
<th>Dye Type</th>
<th>Excitation Range (nm)</th>
<th>Emission Range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue-absorbing</td>
<td>489-516</td>
<td>506-538</td>
</tr>
<tr>
<td>Yellow-absorbing</td>
<td>546-581</td>
<td>570-603</td>
</tr>
<tr>
<td>Red-absorbing</td>
<td>644-680</td>
<td>665-700</td>
</tr>
<tr>
<td>NIR-absorbing</td>
<td>740-785</td>
<td>764-810</td>
</tr>
</tbody>
</table>

When considering a second dye to use alongside a red-absorbing dye such as Alexa 647, the blue-absorbing dyes are worthy of consideration. The large spectral separation between red- and blue- absorbing dyes simplifies the filtering of the two channels. From the available blue-absorbing dyes, Atto 488 has been identified as the best for STORM experiments [31]. A two-color experiment with Alexa 647 and Atto 488 involves three lasers: the 647nm and 488nm lasers for excitation and deactivation of each dye, and the 405nm laser for reactivation. However, in performing these experiments, we observed the 488nm laser to reactivate Alexa 647. This effect is demonstrated in Fig. 4.4. Typically, the dosage of reactivation light is carefully controlled to determine the number of single molecule events per frame. Ideally, in a two-color experiment, one would like to have independent control of the reactivation of each channel. Although the excitation ranges of different dyes are distinct, the reactivation range of the dyes is not distinct and is also relatively broad. Thus, one must balance the reactivation level for both channels with one laser. Control of reactivation in the Alexa 647
channel is compensated in this case where the excitation light for Atto 488 causes reactivation of Alexa 647. For this reason, Atto 488 was eliminated as an option for the second color alongside Alexa 647.

Figure 4.4: Reactivation Crosstalk. The number of single-molecule events of Alexa 647 in a STORM experiment is increased due to the illumination with an ultra-violet reactivation laser. Here, 405nm light is shown to significantly increase the number of events. However, 488 also causes substantial reactivation at low power. Therefore, a two-color experiment in which 488 is used as the excitation for the second channel alongside Alexa 647 results in decreased control of reactivation of Alexa 647.

Of the yellow-absorbing dyes, one promising option is Alexa 568. This dye offers a balance of medium photon yield, favorably low duty cycle, and high photostability and repeatable switching cycles. Atto 565, on the other hand, has a much higher photon yield but lower photostability (meaning the fluorophores bleach faster, and thus fewer survive the initial deactivation sequence before STORM imaging starts). Atto 565 has been used in STimulated Emission Depletion (STED) microscopy [101, 102] and in genetic studies using Fluorescence in situ hybridization (FISH) [103]. However, the low survival fraction results in fewer localization for a STORM reconstruction, and thus decreased image quality. In a situation with a high density of targets, the problem of few localizations is not significant.
Furthermore, a low survival fraction means the problem of high background due to out-of-focus fluorophores is ameliorated. This is an excellent example of the importance of considering specific sample conditions when selecting dyes for STORM. The photon yields of Atto 565 and Alexa 568 are compared in Fig. 4.5. Note the photon counts are in maximum photons per pixel, not photons per fluorophore. For the spatial sampling in this system, the total photons per fluorophore is approximately a factor of three higher. This evidence demonstrates the possibility for Atto 565 to be a superior choice over Alexa 568, depending on sample conditions.

![Figure 4.5: Fluorophore Selection](image)

This histogram shows the high photon yield of Atto 565 as compared to Alexa 568, making it an excellent choice for multi-color STORM experiments in conjunction with Alexa 647.

One further consideration when comparing these two orange dyes is their commercial availability. As described at the beginning of this section, STORM labeling typically uses a two-antibody system. Dye-antibody conjugation is usually carried out by a commercial company, hence the need for a two-antibody system; a company will not have dye-conjugated antibodies for the wide range of experiments being carried out. However, Atto 565 is not commercially available pre-conjugated to a secondary antibody. Instead, Atto 565 is simply available as free dye molecules which must be conjugated to the antibodies before use in a STORM experiment. The additional process of dye conjugation is likely part of the reason Atto 565 is not widely used despite its high photon yield. The conjugation process is not
an insurmountable obstacle. Furthermore, if the conjugation process is carried out in-house, one could potentially eliminate the secondary antibody in the system and thus reduce the physical distance between the target and the dye.

Clearly, the selection of dyes in multi-color STORM experiments is an important and non-trivial step. Dye performance requirements depend on the experimental conditions. Moreover, the large size of SMLM data sets necessitates automated comparison. Full STORM analysis and reconstructions are time consuming when considering multiple dyes. Therefore, to simplify the comparison of dyes, I implemented a fast image processing routine that provides metadata from STORM data. Figure 4.5 is an example of some of the data provided by my custom code. The technique used in calculating STORM metadata is described in Section 4.3.

4.3 Data Processing

Image processing is an integral part of single molecule localization microscopy. The localization techniques in Chapter 3 form the core of the sequence, but there are several other important steps. The image processing pipeline is as follows:

1. Convert from digital units to photons

2. Segment data (i.e. identify point sources)

3. Fine localization

4. Drift Correction

5. Render the super-resolution image

6. Analyze and interpret the results

Steps 1 and 5 are described in Appendices H and I, respectively. The localization methods are those described in Chapter 3. The segmentation approach described here is
modified from [46] to provide information on a large scale regarding fluorophore charac-
teristics including brightness, duty cycle, and background levels. The segmentation code
enabled the decisions regarding fluorophore selection described in Section 4.2. Additionally,
a fiduciary-free drift correction method is presented. When imaging a layer of the sample
several microns from the coverslip, fiducials sitting on the coverslip would be out of focus.
If attached to the cell, the fiducial brightness would be problematic for identifying single
molecules in their vicinity. The drift correction method introduced here takes advantage
of the fact that photoswitchable fluorescent dyes reactivate numerous times, and so the
algorithm pairs repeated reactivations to estimate drift.

4.3.1 Segmentation Processing

The key to super-resolution in STORM and PALM experiments is to control conditions
such that each frame only contains a sparse set of active fluorophores. As shown in Chapter 3,
the condition can be relaxed significantly without sacrificing super-resolution. However,
even scenes with higher numbers of activated fluorophores still have regions with very few
targets. Full 3D localization can be time-consuming, and processing empty areas is inefficient.
Therefore, a pre-processing step identifies local maxima in the data as potential PSFs to be
analyzed. This step is referred to as segmentation.

The primary characteristics that make a good segmentation scheme are speed and a low
rate of false negatives. The segmentation scheme used here performs well in both of these
metrics. Matlab is a matrix-oriented language, and the Matlab routines for filtering are
well-optimized. For this reason, the segmentation code attempts to find local maxima with
a filtering approach. The approach is adapted from Ref. [46]. The first step in the three-step
process is shown in Eq. 4.1, where $\text{uniform}[I, s]$ is a uniform 2D filter operating on the raw
data $I$. The size of the square filter is $s$. A uniform filter is faster than other options such
as Gaussian filters; the choice of the filter shape does not impact results substantially. The
second step is a dilation of $A_1$, as shown in Eq. 4.2. Each pixel is replaced by the largest
value in the surrounding region of size $s_2$. Lastly, the matrices $A_1$ and $A_2$ are compared; if they are equal, that pixel is deemed to be a local maxima. This final step is carried out using matrix logic, as in eq 4.2, where $A_3$ is a matrix containing 1’s at the position of local maxima. Segmentation is repeated for each frame in the full data set.

$$A_1 = uniform[I, s_1] - uniform[I, 2s_1]$$  \hspace{1cm} (4.1)

$$A_2 = max[A_1, s_2]$$  \hspace{1cm} (4.2)

$$A_3 = \begin{cases} 
0, & \text{if } A_1 \neq A_2 \\
1, & \text{if } A_1 = A_2 
\end{cases}$$  \hspace{1cm} (4.3)

A graphical demonstration of segmentation is shown in Fig. 4.6. The raw data contains five images of the DH-PSF. The subsequent images show each step of the segmentation process: the matrix $A_1$ is plotted in b), and $A_2$ is shown in c). The final image shows the raw image in a) with the locations of 1’s in $A_3$ marked. For the case of the DH-PSF, the size $s_1$ of the inform filter is set to the width of the pair of lobes; the result is an estimate for the local maxima at the center of the DH-PSF lobes. However, this same segmentation code also works with a standard PSF. To demonstrate, the second row in Fig. 4.6 shows the same matrices $A_1$ and $A_2$ for the case of a reduced value for $s_1$. When $s_1$ matches the width of a single lobe, the segmentation code identifies individual lobes of the DH-PSF. In the case of the standard PSF, the locations mark the centers of the PSF.

The output of the segmentation code is a list of local maxima which correspond to potential locations of images of the PSF. Additionally, the segmentation code can provide a set of sub-windows surrounding each local maxima. This reduced form of the data is useful as an input to the fine localization methods, which operate on small windows. Further information available from the segmentation code includes the height of the local maxima and the level
Figure 4.6: **Image Segmentation.** The raw image in a) contains five DH-PSFs. The intermediate steps of segmentation are shown in b) and c), where the matrices $A_1$ and $A_2$ are displayed. The raw image is repeated in d) with estimated PSF locations marked with x’s. For the images in b-d), the kernel size $s_1$ was designed to match the DH-PSF. To demonstrate the code can also find standard PSFs, the same steps are shown in e-g) for a value of $s_1$ that is half the original value.

of background surrounding each peak; from these values, the Signal to Background Ratio (SBR) is estimated. The number of maxima per frame provides an approximate sparsity estimate. Figure 4.7 shows an example of metadata provided by the segmentation process. In optimizing experimental conditions, as described at length in Sections 4.2 and 4.1, metadata such as this provides objective comparisons to make decisions regarding fluorophores, buffers, filters, or cameras. In fact, the plots in Figs. 4.4, 4.5, and D.1 are the results from segmentation. The segmentation code is also very fast. As an example, a data set with more than 30,000 frames and a cell nucleus occupying approximately $40 \, \mu m^2$ (270-by-270 pixels) takes more than 40 minutes to acquire, but segmentation took less than 11 minutes on a desktop computer. Speed such as this means the segmentation meta-data is available while the sample is still in place. Experimental conditions can be optimized throughout the course of the imaging process, rather than over the course of several days of acquisition and image processing. This capability was essential in developing the STORM procedure for our
Figure 4.7: **Image Segmentation Metadata example.** Segmentation not only provides information regarding regions that warrant fine localization analysis, but also produces metadata useful for quantifying experimental performance and comparing experimental conditions. Plot a) shows the number of local maxima per frame. The sudden increases correspond to time point when the reactivation laser power was increased, boosting the number of active emitters. A histogram of the maximum values are plotted in b). Note that the values here are maximum counts in the center pixel; the total photons in a PSF is approximately three times larger for the sampling in these experiments. The estimated background histogram in c) and the low-resolution reconstruction in d) also provide useful feedback for experimental design.

4.3.2 Drift correction

The acquisition time of SMLM data can easily approach one hour. Even with a vibration-isolated table, the long acquisition time results in non-trivial drift over the course of the acquisition. The standard approach to drift correction is to include fiducial markers in the sample (e.g. fluorescent beads) which remain on for the duration of the experiment. These can be localized in every frame in post-processing to estimate the drift over time. The positions of emitters are corrected after localization.

There are other methods for drift correction. Some samples do not allow easy inte-
gration of fiducial markers. One fiducial-free drift correction method involves calculating correlations between bright-field images collected at intervals during fluorescence acquisition [93]. A similar approach uses correlations between sub-sets of super-resolution localizations from separate ranges of frames [94]. Such methods are passive and carried out in post-processing. Yet another drift correction option is to measure the deviation in real time and adjust for sample movement actively [91, 92]. In this implementation, the fiducial markers are an array of structures fabricated directly on the coverslip. The markers are imaged on a separate infrared channel in parallel with fluorescence acquisition.

Nikon has commercialized an approach to actively correct for axial drift. In their “perfect focus system,” a light-emitting diode at 870nm is sent into the objective with a lateral offset, resulting in a non-normal reflection at the interface of the sample and the coverslip. The reflection is imaged onto a line CCD. If the sample drifts axially, the position of the reflection on the line CCD shifts; the drift is corrected to keep the position of the reflection stationary. The perfect focus system performs well at keeping the sample in focus over the course of an acquisition. However, the precision is insufficient for 3D localization microscopy measurements and additional drift correction is necessary.

Here, I propose a different method for fiducial-free drift correction. The dyes used in STORM will reactivate numerous times (anywhere from 5 to 100 reactivation cycles, depending on the dye, buffer conditions, and laser powers) [31]. Furthermore, our data suggests the dyes will tend to reactivate in close time points. This drift correction method assumes that if a pair of localizations are close enough in space and time, they are repeated localizations of the same emitter. In a typical STORM data set, many such pairs can be found. The frame-to-frame drift is estimated as the weighted average of all shifts of pairs over the whole data set. The weighting value is the number of photons attributed to each pair of localizations. The examples shown here are for 2D drift correction, but this method is easily extended to three dimensions.

As a verification, the pairwise drift correction was applied to a two-color 2D STORM
data set with 2,700 frames. As can be seen in Fig. 4.8, the drift estimates from the two channels agree quite well. The signal strength in the 647nm channel was lower, which may account for the presence of jitter in the drift estimate for that channel. In a different experiment, the effect of drift correction on a small region of data containing 626 localizations is shown in Fig. 4.9. Before drift correction, the set of point shown in a) have a standard deviation of $\sigma_x = 30.5\text{nm}$ and $\sigma_y = 23.8\text{nm}$ (note the grid pattern due to the 10nm step size of the dictionary). After drift correction, the size of the object reduces to $\sigma_x = 23.8\text{nm}$ and $\sigma_y = 22.1\text{nm}$. The reduction in size in the x-dimension is 22%. Lastly, the pair of images in Fig. 4.10 shows the efficacy of this drift correction method over a period of 11,000 frames. The difference in the objects before and after drift correction demonstrate not only that the method is effective, but also that drift correction is absolutely required for STORM data sets. Without drift correction, there appears to be a pair of long, straight objects with lengths of several hundred nanometers. After drift correction, the objects appear more like clusters with approximate sizes of 100-200nm.

![Figure 4.8: Drift correction in STORM.](image)

The one-dimensional drift for two color channels of the same data set using pair-wise matching result in similar trends.
Figure 4.9: **Drift correction analysis.** Pairwise drift correction reduces the measurable size of an object in a STORM reconstruction. The pre-drift correction collection of points in a) has a standard deviation of 30.5nm and 23.8nm in x and y, respectively. After drift correction, the standard deviation is 23.8nm and 22.1nm in x and y.

Figure 4.10: **Drift correction analysis over time.** Drift correction is demonstrated for a series of 1,272 localizations over 11,000 frames. The pre-drift-corrected points in a) have very obvious smearing due to drift. The drift artifacts are not present in b).

### 4.4 Experimental Results

#### 4.4.1 2D Reconstructions of Viral Objects

Polyomaviruses reproduce by requisitioning the cellular machinery in the nucleus of a host cell. The results presented here are mouse embryo fibroblast cells (MEFs) that have
been infected with Murine Polyomavirus (MPyV). This particular virus does not infect humans, but it is similar to other viruses that can be found in humans. Infection by these viruses can lead to disease and cancer. Furthermore, MPyV is interesting to study to further our understanding of the mechanisms at work during viral production. Viruses have simple genomes; the genome of MPyV has been sequenced, and it is known to contain the information for producing only six gene products. Three of these proteins are responsible for making up the capsid, and the other three proteins are involved in replicating and packaging viral DNA. Antibodies recognizing these proteins can be labeled with STORM dyes in order to study viral reproduction. Viruses have been observed, by electron microscopy, to reproduce in clusters which have been referred to as “virus factories” [58]. The images shown here are the results of a collaboration with the authors from Ref. [58] with the aim of better understanding the spatial arrangement of these virus factories.

Wild Type (C57) MEFs were grown in an imaging dish and infected with MPyV. Viral genome replication and assembly of progeny virions occurs in the nucleus between 24 and 32 hours post infection. Cells were fixed between these times and to reduce nonspecific background, the soluble cytoplasm was extracted prior to fixation. In the images shown here, two different viral proteins have been labeled using primary antibodies conjugated with either Alexa 647 or Atto 565. Viral Protein #1 (VP1) is the major Polyomavirus capsid protein, and T-antigen is a viral protein involved in DNA replication. The super-resolution renderings shown in Figs. 4.11-4.14 have these two proteins as targets. In other experiments, non-viral cellular proteins are labeled; one example is Lamin-B, which is found in the lamina of the cell nucleus. The images in Figs. 4.15-4.16 have VP1 and Lamin-B labeled. See Appendix L for thorough sample preparation methods.

4.4.2 Two-Color Double Helix

The approaches presented in this chapter thus far are relevant for two-color 2D experiments. The additional insight gained through 3D localization microscopy is interesting
Figure 4.11: **Two-color STORM reconstruction.** Infected cells were fixed and imaged with 2D two-color STORM. The Atto 565 dye was attached to the viral protein VP1, shown here in green. The target for Alexa 647 was T-antigen (purple). White boxes indicate regions of zoom in Fig. 4.12. Scale bar: 1μm.
Figure 4.12: **Two-color STORM reconstruction, magnified view.** The regions indicated in Fig. 4.11 are magnified here. As before, the green is VP1 and the purple is T-antigen. Scale bar: 1µm.
Figure 4.13: **Two-color STORM reconstruction, additional view.** A different nucleus from the same sample as Fig. 4.11. The VP1 and T-antigen are green and purple, respectively, as above. White boxes indicate regions of zoom in Fig. 4.14. Scale bar: 1µm.
Figure 4.14: Two-color STORM reconstruction, magnified view. The regions indicated in Fig. 4.13 are magnified here. As before, the green is VP1 and the purple is T-antigen. Scale bar: 1µm.
Figure 4.15: Two-color STORM reconstruction, different targets. In this sequence of images, the green indicates Atto 565, which was attached to VP1 as before. The Alexa 647 was specified to attach to Lamin B, a nuclear protein. This object is shown in purple. White boxes indicate regions of zoom in Fig. 4.16. Scale bar: 1µm.
Figure 4.16: **Two-color STORM reconstruction, magnified view.** The regions indicated in Fig. 4.15 are magnified here. The green is VP1 and the purple is Lamin B. Scale bar: $1 \mu m$. 
for some projects and essential for others. As shown in Chapter 3, the Double-Helix Point Spread Function (DH-PSF) is an excellent option for 3D localization of point sources in single-molecule experiments. All 3D experiments in Chapter 3 invoked only a single color. The work presented here was performed on a user-facility microscope with the aim of making two-color 3D technique more amenable for biologists.

A standard clear-aperture imaging system for 2D SMLM can be converted to a 3D DH-PSF system by including an additional relay system and phase mask before the camera, as in the DH-PSF system in Fig. 3.9 on Page 55. The phase mask for the double helix has a wide but finite bandwidth; maximal diffraction efficiency occurs when the mask is used at the design wavelength. However, since the two color channels are already split as shown in Fig. 4.2, each channel can have a mask designed for the specific wavelength of the dye. The modified system for two-color DH-PSF is shown in Fig. 4.17. As is standard with Nikon systems, the tube lens length is 200mm. In the 2D system, the cameras were located at the image planes; the image is relayed to the new camera system by a pair of 25mm diameter lenses with focal lengths of 50mm. The distance between the image plane and the first lens is one focal length, the pair of lenses is separated by twice the focal length, and the final image plane is one focal length behind the last lens. The DH-PSF phase mask is located approximately in the center of the two lens positions. The assembly containing the pair of relay lenses and the DH-PSF phase mask is referred to as a DH module. These modules were provided by Double Helix LLC.

The physical layout of the optical system shown as a schematic in Fig. 4.17 uses a Nikon Eclipse Ti microscope base, which is an inverted system. In the standard configuration of this base, there is only one turret for dichroics; the excitation light in epi-fluorescence mode enters in the back of the base and is reflected up to the objective. Emission light passes through the single dichroic turret and into the bottom of the base where it can be sent to the eyepiece or one of two side ports, referred to henceforth as the left port and right port. An additional kit raises the sample stage, objective turret, and dichroic turret to make room
Figure 4.17: **Two-color Double Helix.** Modifications to convert the 2D two-color system in Fig. 4.2 for two-color double helix imaging are shown here. The images are relayed with a pair of lenses separated by the sum of their focal lengths, and in between is a double-helix phase mask. The system here uses the same excitation optics and objective as in Fig. 4.2, so those components are omitted here.

for an additional dichroic turret and imaging port. The light reflected by the second (lower) dichroic is sent to the new imaging port in the back of the microscope, referred to as the back port. The systems shown in Figs. 4.2 and 4.17 use the back port and left side port for cameras #1 and #2, respectively.

Unfortunately, when used in this configuration, the images in the back and left port suffer from very reduced fields. The cause is aberrations from the optical system in the microscope base; these aberrations are not problematic when the detector is placed in the plane immediately after the exit port of the microscope base, but relaying the image causes a severe degradation in usable imaging area. Interestingly, these aberrations are only present in the back and left port, but the right port performs well with the identical DH module (see Appendix J for example images).

Conceptually, the layout is identical to the schematic in Fig. 4.17, except that rather than using a dichroic mirror within the turret of the microscope, the color splitting happens outside the microscope base. There are several options in designing this system. One option is to use a color splitter that divides the detector of a single camera into two channels, thus avoiding the need of an additional camera. This option is especially interesting for a
camera such as the sCMOS, since said camera has a very large number of pixels (2048-by-2048). However, the sCMOS also has smaller pixels, necessitating an objective with lower magnification. A second option is to use a color splitter that sends the light from each channel to separate cameras. In this configuration, the DH module could be installed before the splitter, and only one module is required. Alternatively, each channel could use a DH module for the specific wavelength in that channel; the modules would be positioned between the color splitter and the camera. The observations presented here suggest that either arrangement will provide a flexible platform for two-color 3D super-resolution experiments.

4.5 Discussion and Conclusion

We have presented a combination of tools that enable multi-color super-resolution imaging in a thick sample. The first aspect of this endeavor involved selection of a pair of fluorophores that perform well in these sample conditions. The selection process was aided by the processing technique described here that permits fast evaluation of fluorophore performance on a large scale. The drift correction method reported here provides a fiducial-free method for estimating the sample shift over the course of a long multi-color SMLM acquisition. The resulting super-resolution image renderings provide a view into the reproduction of the Murine Polyomavirus in mammalian cells. Lastly, the prospect of two-color 3D super-resolution on a modified commercial system is explored, and several options are described.

The results shown here are the product of a long collaboration involving experimental optimization and image processing development. Although the operating goal involved a specific project that requires two-color STORM in a thick sample, the true goal was much broader than that. Single molecule localization microscopy is a powerful imaging paradigm. The potential impact of this super-resolution technique has yet to be fully realized due to the multiple compounding hurdles of biochemistry, optics and measurement, and image process-
ing. The results in the final section of this chapter are not simply interesting observations regarding viruses; they are meant to be demonstrative experiments to incite further interdepartmental collaborations and to assist in realizing the full potential of super-resolution microscopy.
Chapter 5

Conclusions and Future Work

The 2014 Nobel Prize in chemistry was awarded to Eric Betzig, Stefan Hell, and William Moerner for their work in developing super-resolution fluorescence microscopy. While recognition such as this signifies the importance of super-resolution microscopy, I believe the major breakthroughs resulting from super-resolution techniques are yet to come. Interesting observations have been made, but several significant advancements in the state of the art are still under development.

One such advancement is the need for faster imaging. The motivation for enhanced imaging rates is partially in response to the notoriously long acquisition times; faster acquisition means more regions of interest can be examined. Some difficult questions require large databases of reconstructed images to arrive at a conclusion. More importantly, reduced data acquisition times means living cells dynamics are observable. Video-rate SMLM could provide insight into fast dynamics; similarly, slow dynamics could be observed by tracking the same object of interest over time and collecting super-resolution images at several time points over the course of a process of interest.

Another improvement is the transition from 2D to 3D measurements. Very few processes occur in two dimensions, and a 2D projection of a 3D environment suffers from a dearth of information. Consequently, quantitative measurements from 2D experiments are less certain. There are several options for obtaining 3D localization information, but all im-
implementations impose additional sample constraints regarding thickness, background level, and labeling density. These disadvantages can be overcome with better fluorophores, novel optical architectures, and more robust processing techniques.

The final component that will enable an abundance of discoveries to come out of super-resolution microscopy is the matter of accessibility. Successful experiments require expertise in biochemistry for sample preparation, specialized optical systems, and efficient computational routines. Commercially available PALM and STORM fluorophores lower the barriers to entry into the field, and complete super-resolution systems simplify the process of optical system design and construction. Several open-source and proprietary image processing packages further broaden the base of potential users. These tools will greatly assist in 2D single-color experiments. Refinements such as multi-color, 3D, or thick samples require custom modifications to standard approaches to sample preparation, optical system design, and image processing.

5.1 Quantum Dots in Microscopy

The quality of super-resolution reconstruction images depends primarily on the localization precision, which is in turn defined by the emitter intensity. The incredible brightness of quantum dots in comparison to typical fluorescent dyes and proteins makes them excellent candidates for localization microscopy. In the work presented in Chapter 2, the interesting blinking properties of QDs are shown to be useful as a resolution mechanism for classically unresolved QD. The ICARUS algorithm combines the temporal information from blinking with spatial information regarding the point spread function. The algorithm furnishes an estimate of the number of emitters in the scene, the images and locations of each emitter, and the time signal associated with the QD blinking. Dense clusters of QDs can be resolved with this approach; simulations indicate as many as five QDs within a single diffraction-limited spot can be resolved and localized with precision better than 20nm. An
experimental demonstration confirms the capability. Additionally, analysis of the outputs from ICARUS yielded observations regarding the blinking behavior of closely spaced QDs at unprecedented length scales. These observations confirmed the assumption that neighboring QD are truly independent.

5.2 Sparse Reconstructions for Localization

The issue of data acquisition time for SMLM is addressed in Chapter 3. Similar to the ICARUS algorithm in Chapter 2, the approach here involves localization algorithms that can tolerate overlapping images of the point spread function. The algorithms in Chapter 3 are specifically designed with 3D PSFs in mind. Although PSFs such as the double-helix or the astigmatic both convey precise depth information, the drawback for both cases is a larger PSF that further limits the number of active fluorophores per individual frame of SMLM data. However, even apparently dense scenes have a high degree of mathematical sparsity. The sparsity-based localization techniques described in this chapter can resolve and localize ten times more emitters per frame than previous double-helix localization algorithms. Lastly, localizations obtained from a biological sample were analyzed to produce a 3D quantitative measurement of the radius of anti-body labeled microtubules.

5.3 Expanding Super-resolution in 3D

The final approach to improving super-resolution presented in this Dissertation involves a detailed evaluation of the biological, optical, and computational means for expanding the capabilities of SMLM. The computational tools presented here enable fast optimization of experimental design to add multi-color STORM capabilities to the existing SMLM. Analysis and testing of the microscope optical system with double-helix modules provides a path for further expanding the multi-color system to 3D. The ultimate goal of the work presented in Chapter 4 is to push the boundaries of SMLM while also making these advanced tools
available for interdisciplinary collaborations. A project involving viral reproduction in mammalian cells offered the opportunity to demonstrate the improvements in a new application.

5.4 Future Work

5.4.1 Quantum Dots

Although QDs have many favorable characteristics for localization microscopy, they have yet to be widely implemented in biological applications. One problem is the current incompatibility between the photoswitching methods of SMLM and the random blinking of QDs. The work presented in Chapter 2 provides a means for resolving and localizing as many as five QDs in a diffraction-limited spot. Given the wavelength and numerical aperture of the system in that demonstration, the equivalent emitter density is greater than 26 emitters/µm². However, demonstrations of the ICARUS algorithm assumed some space between neighboring clusters. A more conservative estimate of the maximum recovery density is closer to 6 emitters/µm². For comparison, standard localization methods tolerate emitter densities of approximately 1 emitters/µm², and other high-density 2D methods with photoswitchable dyes and proteins have a maximum recovery density of ∼8-10 emitters/µm² [48, 51]. The primary advantage of photoswitchable dyes is the low duty cycle, which creates the condition that most emitters are inactive in any given frame. For Alexa 647 in a βME buffer, the duty cycle is 0.0012 [31], meaning fewer than 1 in 800 dye molecules are in the active state. Thus, the density of localization in reconstructions from dye experiments can be well over an order of magnitude higher than a QD experiment.

The evolution of fluorescent proteins and dyes has been in development for decades, beginning perhaps with initial demonstrations of immunofluorescence in 1942 [6]. Quantum Dots are much newer, with significant advances in biological applications only coming in the last 15 years [104]. Further development of QDs as biological probes is under way. In one case, researchers fabricated a QD-dithienylethene complex which allows for optical regulation
of QD emission [105]. Biochemical modulation of QD characteristics is another avenue that might lead to expanded QD applications. For example, a novel coating for QDs provides enhanced cellular uptake by live cells, low cytotoxicity, and the interesting property that the formation of a DNA-QD complex resulted in reduced QD intensity [106]. The resulting QD system can potentially be used as a nonviral gene delivery method. For tracking experiments, the blinking is problematic. However, better understanding of blinking behavior has enabled a reduction of blinking activity, leading to studies of membrane dynamics, synapses, and intracellular processes [107]. Innovations such as these will further expand the applications of QDs, allowing them to fulfill their potential as bright probes for fluorescence microscopy.

5.4.2 Localization Algorithms

The sparsity-based approaches for localizing overlapping emitters presented in Chapter 3 utilized two different algorithms for performing the reconstruction of individual frames of localization microscopy data. These two algorithms were selected because they offered very different results in regards to computational complexity and maximum recoverable density of emitters. There are other mathematical algorithms that could fit into the same paradigm of dense emitter fitting and could potentially offer enhanced performance in either computation time, maximum density, or both. For example, orthogonal matching pursuit [108] may improve the resolution capability of standard matching pursuit for closely-spaced images of PSFs without significantly increasing computational complexity. Modifications to the convex optimization algorithm have been proven to be useful in compressive sensing problems in which the dictionary is highly coherent and redundant [71], as is the case here. Yet another modification to the convex optimization approach maintains similar performance in localization density but performs the calculation in one or two orders of magnitude less time than an implementation using CVX [49]. Additionally, widespread interest in compressive sensing has inspired researchers from the applied and pure mathematics disciplines to propose completely new algorithms that address common problems in practical experiments [109].
As computational capabilities continue to increase, new algorithms will play an important role in advancing localization microscopy capabilities.

The typical image processing approach in SMLM involves many similar calculations on small image patches. This type of problem is ideally suited for execution with a Graphics Processing Unit (GPU), which offers substantial decreases in computation time for appropriate problems. Several researchers have implemented high-density localization algorithms on GPUs, resulting in speed increases of 1-3 orders of magnitude [46, 51, 54].

5.4.3 Localization Microscopy in Thick Samples

The effect of objective numerical aperture in microscopy creates a conflict between resolution and depth of field. The high-NA objectives used in localization microscopy have DOFs of 400nm which can be extended up to 2µm with engineered PSFs. The thickness of biological objects might be several times the DOF, as is the case in Chapter 4. Therefore, the reconstructions obtained from standard epi-fluorescence illumination only contain information from a slice of the object. Additionally, the out-of-focus portion of the object is still excited, resulting in increased background and thus decreased localization precision. The problem of background can be addressed by changing the illumination scheme such that only the in-focus volume of the sample receives excitation light. Possible optical systems that achieve optical sectioning include light sheet illumination [84, 85], Bessel beam plane illumination [86, 87], two-photon absorption [4, 88], and temporal focusing [89, 90]. These approaches vary widely in cost, optical complexity, and performance. However, they all offer improvements over epi-illumination in which the entire depth of the sample receives excitation light. Furthermore, optical sectioning offers the opportunity to collect a super-resolution reconstruction of the full volume by scanning the sample through the excitation region.
Bibliography


Appendix A

Glossary

This is a glossary of terms and abbreviations used in this Dissertation.

- 2D - two-dimensional
- 3D - three-dimensional
- 3DDS - three-dimensional DAOSTORM
- $\beta$ME - $\beta$-mercaptoethanol
- $\lambda$ - wavelength
- (s)CMOS - (scientific) Complementary MetalOxideSemiconductor (referring to the camera architecture)
- CRLB - Cramér Rao Lower Bound
- DH - Double Helix (also, DH-PSF for Double Helix Point Spread Function)
- DHPSF-A - The analytic version of the Double Helix Point Spread Function
- DHPSF-N - The numerically optimized version of the Double Helix Point Spread Function
• DOF - Depth Of Field
• EMCCD - Electron Multiplying Charge-Coupled Device
• FWHM - full-width half-max
• GFP - Green Fluorescent Protein
• GPU - Graphics Processing Unit
• ICA - Independent Component Analysis
• ICARUS - Independent Component Analysis for Resolution Using Spatial information
• LUT - Look Up Table
• MEA - β-mercaptoethylamine
• MEF - Mouse Embryo Fibroblast (cells)
• MLE - Maximum Likelihood Estimator
• MPyV - Murine Polyomavirus
• MTF - modulation transfer function
• \( n \) - index of refraction (unless otherwise noted)
• NA - Numerical Aperture
• PA-GFP - Photo-Activatable Green Fluorescent Protein
• PDF - Probability Density Function
• PSF - Point Spread Function
• QD - Quantum Dot
• QE - Quantum Efficiency

• RV - Random Variable

• SBR - Signal to Background Ratio

• SMLM - Single Molecule Localization Microscopy

• SOFI - Super-resolution Optical Fluctuation Imaging

• STED - STimulated Emission Depletion

• TIF - Tagged Image File

• TIRF - Total Internal Reflection Fluorescence

• UV - Ultra Violet

• VP1 - Viral Protein #1
Appendix B

Mutual Information

The issue of independence for QDs is of paramount importance; if neighboring QDs are not independent in their blinking behavior, a fundamental assumption of ICARUS is violated and the accuracy of the result cannot be guaranteed. As a test for independence, I use the metric Mutual Information (MI), as described in Section 2.6 on Page 26. In this appendix, I present additional details regarding the calculation of MI from discrete data.

In a related work, classically resolvable QDs were shown to have correlated blinking behavior [64]. The metric applied in that paper involved correlations of time signals from QDs. A correlation implies dependence, but uncorrelated random variables (RVs) may not be independent. Therefore, correlations are only useful for disproving independence but not for proving independence, and a different metric is required. MI is one such metric.

Before describing how to calculate MI, I will first present a definition of independence. Two RVs are independent if the realization of one RV does not affect the probability distribution of the other RV. Alternatively stated, knowledge of one RV does not provide any knowledge of the other RV. This can be written as:

\[ p(x \mid y) = p(x), \] (B.1)

where \( p(x) \) denotes the probability density function (PDF) of the RV \( X \), and \( p(x \mid y) \) is the conditional distribution of \( X \) given \( Y = y \). From this statement, it directly follows that the
joint density of $X$ and $Y$ must be a separable function that is the product of the marginal densities. Mathematically:

$$p(x, y) = p(x)p(y),$$

(B.2)

where $p(x, y)$ is the joint density and $p(x)$ and $p(y)$ are the marginal densities [110]. Thus, a test of independence can test the validity of this statement. The calculation of MI provides such a test since by definition:

$$MI(X; Y) = \frac{1}{\log(N)} \sum_{x \in X} \sum_{y \in Y} p(x, y) \log \left( \frac{p(x, y)}{p(x)p(y)} \right),$$

(B.3)

where $N$ is the number of samples of the RVs. The factor $\frac{1}{\log(N)}$ is introduced for normalization, such that a score of $MI = 0$ indicates independence, whereas $MI = 1$ indicates complete dependence. However, we do not have access to the functions $p(x, y)$, $p(x)$, or $p(y)$; we must estimate them from binned samples from the microscopy data. Since we are trying the populate the 2D histogram $p(x, y)$ with as few as 500 values, the binning must be rather coarse. As a consequence, sometimes a pair of binned values $x_i$ and $y_j$ appear to occur multiple times, when in fact the more continuous values for $x$ and $y$ were simply occurring at values within the bin edges of $x_i$ and $y_j$. The corresponding MI score for estimated PDFs based on a limited number of binned samples is non-zero. An example of the binning procedure for experimental data from Chapter 2 of two raw pixels of blinking QDs is shown in Fig. B.1. The histograms in a) and b) approximate the marginal densities $p(x)$ and $p(y)$, whereas the 2D histogram in c) approximates the joint density $p(x, y)$. Note that all independence calculations are performed on signals that have been converted to be zero-mean, unity-variance signals, hence the arbitrary units of intensity in the histograms.

To justify the non-zero threshold for the mutual information score that indicates independence, 5000 independent RVs were generated. These were then binned according to the same procedure for measuring mutual information of time signals of blinking quantum dots. The MI scores were distributed as in Fig. B.2 a). The mean score was 0.088 with a standard deviation of 0.00395. The range of possible MI scores are between zero and one;
Figure B.1: **Probability Density Function binning.** Histograms of two experimental quantum dot blinking signals are shown in a) and b), and the combined histogram of the two signals is shown in c). These histograms are the estimates for $p(x)$, $p(y)$, and $p(x, y)$, respectively.

This range is shown graphically in b). Therefore, the imperfect binning procedure results in a non-zero MI score for truly independent RVs. The null hypothesis is that two RVs are independent. If the MI score is greater than 0.095, the p-value is $p < 0.05$, indicating the null hypothesis ought to be rejected. Conversely, an MI score less than 0.095 indicates the RVs are independent.

Figure B.2: **Mutual Information (MI) score threshold.** A sequence of 5000 random variables was generated and their MI scores were calculated from binned samples. The resulting MI scores for these truly random variables is shown in a). The same score with respect to the full range of possible scores for MI is shown in b). The interpretation of these results, including the independence threshold, is shown in c).
Appendix C

Dictionary Normalization

An orthonormal dictionary, or basis set, is a convenient choice for expressing an image. However, SMLM requires sub-pixel localization; dictionary-based localization methods such as those presented in Chapter 3 must use overcomplete dictionaries. Therefore, the dictionaries are not orthogonal. However, the normalization of the dictionaries can have a considerable effect on the outcome of sparsity-based techniques. In this appendix, I present results from experimental data demonstrating the effect of incorrect normalization of dictionaries.

With an orthogonal basis set $B$, the image $x$ can be simply expressed as the sum of individual basis elements $b$:

$$x = \sum_{b \in B} \frac{\langle x, b \rangle}{\|b\|_2} b,$$

where $\langle x, b \rangle$ is the projection (dot product) of $x$ onto $b$, and $\|b\|_2$ is the L2-norm of $b$. For an orthonormal basis set, $\|b\|_2 = 1$ and the expression simplifies to

$$x = \sum_{b \in B} \langle x, b \rangle b.$$

(C.2)

The convenience of an orthonormal basis set is now evident; coefficients of basis elements are found by simply projecting the image onto the element. Furthermore, conversion to a new basis set can be performed with a single matrix multiplication.
For a non-orthogonal basis set, decomposing an image into a new basis set involves more
difficult techniques (as described in Chapter 3). In Matching Pursuit (MP), for example,
the sparse basis elements that are present in the scene are identified in an iterative process.
For each basis element present in a scene, there are three relevant values to keep track of:
the projection \( p \), the number of photons \( N \), and the \( \ell_1 \)-normalized coefficient \( c_{L1} \). The
calculation and significance of these values are described here.

The basis set is \( \ell_2 \)-normalized (\( \|b\|_2 = 1 \)). The projection \( p \) is simply a dot product
with the image and the dictionary element:

\[
p = \langle x, b \rangle.
\]  
(C.3)

The projection \( p \) is a useful value for reconstructing the image from the dictionary elements
that compose that image. Specifically, if an image contains two emitters, the scene can be
reconstructed as:

\[
x = p_1 b_1 + p_2 b_2.
\]  
(C.4)

The projection is used when finding the basis elements that make up a scene; once an element
\( b_k \) has been determined to be present in the scene, a residual image \( x_{k+1} \) is calculated by
subtracting the projection from the previous residual image:

\[
x_{k+1} = x_k - p_k b_k.
\]  
(C.5)

For determining if a stopping criterion has been met, we must also calculate the number
of photons corresponding to this projection. The photon count \( N \) is simply the sum of the
portion of the image accounted for by a particular basis element:

\[
N = \sum_{\text{pixels}} x_{\text{emitter}} = \sum_{\text{pixels}} \langle x_k, b_k \rangle b_k = p_k \sum_{\text{pixels}} b_k = p_k \|b_k\|_1,
\]  
(C.6)

where \( \sum_{\text{pixels}} \) is the sum over all pixels and \( x_{\text{emitter}} \) is the portion of the full image \( x \) that
originates from a particular emitter (excluding other emitters in the scene and background
noise).
The third relevant value is the L1-normalized coefficient $c_{L1}$. This is identical to the projection $p$ if the dictionary were L1-normalized ($||b||_1 = 1$). This can be calculated from $p$:

$$c_{L1} = \frac{p}{||b||_1}.$$  

When searching for the most significant basis element $b_k$ in the $k^{th}$ iteration of MP, we must select the basis element with the maximum value for $c_{L1}$, and not $p$ or $N$.

The effect of selecting the maximum value from the space of $p$ or $N$ results in artifacts which cause the MP algorithm to preferentially select basis elements with a high L1 value. Consequently, the reconstructions exhibit a grid-like artifact where localizations tend to lie on the borders between pixels, and especially at the intersections of four pixels. As a demonstration, 1000 completely random 8-by-8 pixel scenes were generated and projected onto an L2-normalized dictionary of 2D PSFs with 1/16$^{th}$ of a pixel steps between dictionary elements. The value for $p$, $N$, and $c_{L1}$ for each dictionary element is summed over all 1000 random scenes. The range of values for $p$, $N$, and $c_{L1}$ is shown graphically in Fig. C.1 a), b), and c), respectively. The standard deviations for the three values are 1808, 7087, and 129. The conclusion to be drawn from this set of figures is that if one selects a basis element $b_k$ during an iteration of MP based on the maximum value of $p$ or $N$, the brighter basis elements in Fig. C.1 a) and b) will be more likely to be selected. On the other hand, if one finds the maximum from $c_{L1}$, there will be no preferential selection of any dictionary elements. To situate the grid-pattern in Fig. C.1 a) and b) with respect to the pixels, Fig. C.2 shows the edges of the pixels as blue lines painted on top of an image of the L1-norm of an L2-normalized dictionary.

The grid-like artifact due to improper dictionary element selection can also be seen in reconstructions from experimental data. A 30,000-frame data set was processed using three versions of MP which selected dictionary elements based on the maximum value for $p$, $N$, or $c_{L1}$. Each method found between 1.1 and 1.4 million emitters in the data set. Fig-
Figure C.1: **The effect of dictionary normalization with random scenes.** The images in these three scenes show the values of $p$, $N$, and $c_{L1}$ for each basis element of an L2-normalized dictionary when projected onto 1000 random scenes. Only the image in c) displays no preferential selection of any particular dictionary elements.

Figure C.2: **Dictionary normalization with respect to pixel edges.** This image shows the L1-norm of an L2-normalized dictionary; the blue lines indicate the pixel edges. Note that no elements are included in the dictionary that are located within $1/2$ a pixel from the edge of the 8-by-8 scene.

Figure C.3 shows the frequency with which particular dictionary elements were selected. The grid pattern in a) and b) reflect the tendency to incorrectly select dictionary elements that lie at intersections of pixels. Figure C.3 c), on the other hand, shows no such grid pattern. There is, however, a notable predisposition towards selecting elements that lie within the central pixel of the dictionary. This is simply the result of a segmentation algorithm that performs well at identifying and cropping local maxima in the raw data, resulting in many small patches in which the correct result is in fact within the central pixel of the dictionary. As further evidence of the importance of using $c_{L1}$ when selecting dictionary elements, Fig. C.4 show a close-up of localizations from the same data set. The grid artifact causes
substantial degradation of the quality in a) and b) to the point where the reconstructions are of questionable value. The localizations in c), on the other hand, provide interesting and potentially useful super-resolution information.

Figure C.3: The effect of incorrect MP approaches on experimental data. The images in a)-c) indicate the frequency with which dictionary elements are included in the solution when Matching Pursuit selects maxima based on $p$, $N$, and $c_{L1}$, respectively. The colors indicate the number of times a particular dictionary element is chosen out of the 1.1 to 1.4 million emitters in the data; the colors are on a log scale. Only the MP method using $c_{L1}$ avoids the grid artifact that preferentially selects dictionary elements at pixel intersections.

Figure C.4: Grid artifacts in super-resolution reconstructions. A close-up of super-resolution localization from experimental data shows the intolerable grid artifacts from incorrect MP algorithms. The scenes in a)-c) correspond to Matching Pursuit approaches based on $p$, $N$, and $c_{L1}$, respectively.
Appendix D

Buffer Selection

Dye characteristics depend on the thiol and buffer used during STORM imaging. This effect is especially relevant in multi-color STORM because different dyes respond to buffer composition independently. The options for thiols is less extensive than the choice of dye: the options are $\beta$-mercaptoethanol ($\beta$ME) and $\beta$-mercaptoethylamine (MEA). The effect of thiol choice for a two-color experiment using Alexa 647 and Alexa 568 is shown in Fig. D.1. For both fluorophores, $\beta$ME results in more high-count emissions. However, in the case of Alexa 647, this actually results a reduced signal-to-background ratio. The thiol choice also affects the duty cycle; here, an increased photon count is offset by an increased duty cycle, resulting in more out-of-focus fluorophores and thus increased background.
Figure D.1: **Buffer Selection.** The effect of thiol is demonstrated for a two-color STORM experiment using Alexa 568 and Alexa 647. For both dyes, the photon yield is higher with βME (a,c). For Alexa 568, higher photon counts provides enhanced signal-to-background ratios (b). However, Alexa 647 experiences a higher fraction of activated fluorophores with βME, which results in increased background (d).
Appendix E

Laser Excitation

Before an acquisition of STORM data begins, the majority of the dyes in the scene are in an active state. The population of fluorophores must be deactivated to a sufficient extent so that individual dyes can be resolved. Deactivation requires high intensity of the excitation laser and the presence of a quenching agent, as described in Section 4.2. Depending on the dye and the laser intensity, deactivation may require anywhere from 30 seconds to 10 minutes. If the laser intensity is too low, proper deactivation may not occur at all; many of the dyes will remain in an active state rather than converting to the temporary dark state.

For the experiments presented in Chapter 4, the laser illumination system is an Agilent MLC 400 monolithic laser combiner. In this commercial system, four laser are combined and coupled to an output fiber. The wavelengths are 405nm, 488nm, 561nm, and 647nm. Switching of the lasers is achieved with acousto-optic tunable filters within the laser system; switching can be controlled by the camera so that lasers are delivered only during data acquisition and proper deactivation and reactivation cycles occur. The output intensity from this system is sufficient for timely deactivation. In contrast, initial experiments were performed on a system with confocal and wide-field capabilities and a less powerful laser system. The lasers on the confocal (“A1R”) system were insufficient for deactivation. A comparison of the laser power exiting the high-NA objective for super-resolution is shown in Fig. E.1. The N-STORM has the additional advantage that the red laser is at 647nm,
rather than 638nm. By using a laser with a wavelength that coincides with the excitation peak of the most popular STORM dyes, more of the higher power of the N-STORM system can be absorbed by the dyes.

![Laser Power for STORM](image)

**Figure E.1: Laser Power for STORM.** Dye deactivation requires high intensities at the excitation wavelength of the dye. The N-STORM, which provides sufficient power, is compared with a system incapable of deactivating populations of dye for STORM imaging.

The output fiber of the laser combiner connects to the TIRF illuminator. For standard wide-field epi-fluorescence, the system is designed such that the illumination area is slightly larger than the EMCCD’s 81.92µm field-of-view. Additionally, the TIRF illuminator contains a lens that concentrates the laser spot on a smaller area. Higher intensity comes at the cost of less uniform illumination across the full field of view. A measurement of the illumination area with and without the concentrator lens is shown in Fig. E.2. Each image is a montage of a 25 images in a 5-by-5 tiled pattern. The montages were created after several minutes of high intensity illumination of a STORM sample. These images provide the dual purpose of showing the effectiveness of deactivation while also providing an estimate of the illumination areas with and without the concentrating lens. Using these areas, the estimated laser intensity for all possible illumination conditions is calculated; the results are tabulated
in Table E.1.

Figure E.2: **Laser spot sizes.** These montage images show the area of illumination. Without a concentrating lens, the radius of illumination is approximately 140 µm, as seen in a). With the concentrating lens in place in b), the illumination radius is reduced to approximately 67 µm. The camera field-of-view is 81.92 µm; the stitching lines between adjacent images can be seen here.

Table E.1: **Laser Intensity for STORM.** This table provides the laser intensity at the focal plane for the two microscope systems where STORM experiments were performed. Each microscope also had a removable concentrator lens which focused the laser into a smaller region.

<table>
<thead>
<tr>
<th>System</th>
<th>405nm*</th>
<th>488nm*</th>
<th>561nm*</th>
<th>638/647nm*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confocal (A1R)</td>
<td>5.6</td>
<td>11.3</td>
<td>20.1</td>
<td>12.6</td>
</tr>
<tr>
<td>Confocal with concentrator</td>
<td>24.4</td>
<td>49.2</td>
<td>87.7</td>
<td>55.1</td>
</tr>
<tr>
<td>N-STORM</td>
<td>9.3</td>
<td>47.3</td>
<td>52.7</td>
<td>145</td>
</tr>
<tr>
<td>N-STORM with concentrator</td>
<td>40.5</td>
<td>206</td>
<td>230</td>
<td>634</td>
</tr>
</tbody>
</table>

* All intensities are shown in W/cm².
Appendix F

Spectral Filtering

With any fluorescence imaging system, one must pay careful attention to proper filtering to maximize signal and minimize background. In the case of STORM, the problem is exacerbated because excitation powers are very high and light levels at the detection wavelength are much lower. A multi-color STORM puts even more demands on spectral filtering. Figure F.1 shows the spectral situation for the particular two-color experiment using Alexa 647 and Atto 565, including the three lasers for excitation/deactivation and reactivation.

Figure F.1: Spectral View of Two-Color STORM. This plot summarized the spectral situation of a two-color STORM experiment. There are three lasers: the 405nm laser for reactivation, and the 561nm and 647nm lasers for excitation/deactivation of Atto 565 and Alexa 647, respectively. Also shown here are the excitation and emission spectra for those two dyes.
The excitation wavelength for Alexa 647 is higher than the emission wavelength of Atto 565. Indeed, many combinations of dyes will have an intertwining of excitation and emission. Therefore, the dichroic mirror for separating the excitation light from the emission light must have multiple bands, rather than a simple long-pass dichroic. The other option is to switch the dichroic between excitation of each dye, but the additional time for mechanical movement of a dichroic turret is prohibitive for the already long STORM acquisition times. The system described here has a quad-band dichroic which reflects all four laser lines of the system and transmits the emission bands of most fluorophores. The transmission spectrum for the quad-band is shown in Fig. F.2. The dichroic cube containing the quad-band dichroic also holds an emission filter with matched transmission bands to further reject the high-intensity excitation light. After the emission of Atto 565 and Alexa 647 have been filtered by the quad-band dichroic and emission filter, the remaining spectrum of each dye is shown in Fig. F.2.

Figure F.2: Color Filters for Two-Color STORM. The microscope system is equipped with a quad-band dichroic to permit excitation and reactivation using any combination of the four lasers (405nm, 488nm, 561nm, and 647nm) while simultaneously allowing transmission of the emission light from most fluorescent probes. The spectra of Atto 565 and Alexa 647 after passing through the quad-band dichroic and emission filter are shown in b).

The quad-band dichroic is capable of effectively separating the excitation light from the emission light for this two-color experiment. Potentially, one could use a single camera and a quad-band dichroic and emission filter to image multiple dyes, and image both colors onto the same camera in separate frames by alternating the excitation lasers. However, the
excitation band fluorescent dyes is broad. Alexa 647 can actually be excited by the 561nm laser, albeit at only approximately 10% of the maximum excitation efficiency; I refer to this problem as “excitation cross-talk.” During a frame in which the orange-emitting Atto 565 is being excited and imaged, the red emission from Alexa 647 contributes substantial background. Figure F.3 demonstrates the effect of excitation cross-talk. The sequence shows a pair of images (a and b) collected using only the quad-band dichroic and quad-band emission filter. While the red (Alexa 647) channel contains many fluorophores on a relatively dark background, the orange (Atto 565) channel exhibits intolerable background levels. The third image in the sequence shows the identical scene as b), but with an additional single-pass-band filter to eliminate any emission light from Alexa 647. High background lowers localization precision. Therefore, the two channels must be further separated spectrally with specific bandpass filters to prevent red light from being detected in the orange channel.

Figure F.3: **Excitation Cross-talk.** A biological sample containing both Alexa 647 and Atto 565 was imaged using only the quad-band dichroic and quad-band emission filter; the images for the Alexa 647 and Atto 565 channels are shown in a) and b), respectively. The high background in b) is due to the 561nm laser exciting the Alexa 647 dyes. An additional bandpass filter on the Atto 565 channel can eliminate this background, as shown in c).

One option for effective channel separation is to use an automated filter wheel to move filters in time, but this is slow. Alternatively, one can separate the colors onto two different cameras (or the same half of one camera). The dual-camera approach is easily implemented with the microscope base currently in use. The base contains an additional dichroic turret
and camera port; the system layout is shown in Fig. 4.2. The second dichroic cube contains a single-edge dichroic mirror that reflects all wavelengths below 646 nm. Additional emission filters for each specific channel further ensure unnecessary background is eliminated.
Appendix G

Camera Comparison

G.0.4 Quantum Efficiency

The final component of the optical system is the camera. There are two types of cameras with sufficient sensitivity for the low light levels in SMLM experiments: EMCCDs and CMOS. In the past, EMCCDs were the standard for low-light applications. However, newer generations of CMOS are improving in terms of read noise, quantum efficiency (QE), pixel count, and speed; CMOS is now a viable alternative for some situations [54, 111]. The right port of the microscope is equipped with a Hamamatsu Orca Flash 4.0 CMOS camera. When compared with the Andor iXon Ultra EMCCDs on the left and back ports of the microscope, the CMOS has higher read noise of 1.9 electrons per pixels (RMS). The EM gain in the EMCCDs reduces the read noise to less than one electron. As a result, the EMCCD performs better for low light levels and very low background levels. As the signal and/or background increases, shot noise and background noise start to dominate, and the difference in read noise is less important. Regardless, the reduced QE of the CMOS cannot be ignored, particularly at the emission wavelength of Alexa 647. At 670nm, the EMCCD has a QE of appx. 92%, whereas the CMOS is appx. 66%. The chart in Fig. G.1 shows the approximate QE curves for the two cameras. See Section G.0.5 for an experimental comparison of the localization precision of these two cameras for different signal and background levels.
Figure G.1: Camera quantum efficiency. The quantum efficiency of the EMCCD is higher than the CMOS across the visible spectrum.

G.0.5 Experimental Localization Precision

To determine the observable localization precision on the experimental system where the STORM experiments take place, I collected a series of data sets of 100nm polystyrene beads emitting in the deep red (680nm). A single data file consisted of 500 frames of in-focus beads; five data files were collected at different excitation intensities ranging from 3% to 20% of full laser power. To create the condition of increased background, another set of similar data was collected with the room lights on. However, due to the increased background, the excitation intensity was increased; two data sets were collected with the laser power at 15% and 25%. Lastly, all measurements were repeated with a CMOS camera and 60x 1.49 NA objective in addition to the EMCCD with the 100x 1.49 NA objective. The CMOS camera has a pixel pitch of 6.5 $\mu m$, whereas the EMCCD pitch is 16 $\mu m$, hence the different magnification of the objectives.

Ten beads were selected from each data file and localized in every frame. Drift correction was accounted for by looking at the position of the set of ten beads over time and averaging over all ten beads. The localization precision is quantified as the standard deviation of the 500 points for each bead. Additionally, the number of photons per bead was also quantified. The result is a set of 50 data points for each camera in the low background case.
and 20 data points for each camera in the high background case; those points are plotted in Fig. G.2. The horizontal error bars show the standard deviation of the number of photons. The values for the photons have been corrected to account for the QE of the cameras. Finally, a line was fit to each camera for both levels of background. The fit line has a $1/\sqrt{N}$ character, and the fit is in a least-squared sense.

![Graphs showing camera comparison for localization precision.](image)

**Figure G.2: Camera Comparison for Localization Precision.** Experimental localization precision as a function of intensity level for fluorescent beads is plotted here. For low background levels, 50 beads were analyzed over 500 frames, and the results are plotted in a) for two different cameras. The plot in b) is the same data as a), but zoomed in on the low intensity region. The same process was repeated with high background levels and shown in c). Again, the plot in d) is the identical data as in c), but with the abscissa limited to low intensity values for comparison with b).

The general behavior of the localization precision is as predicted. The best localization precision was better than 2.7nm, and that was with the room lights on. The most interesting
observation one can draw from these measurements involves the camera comparison. The main advantages of the EMCCD are the increased QE and reduced read noise. At low light levels and low background levels, these traits are of paramount importance. Not surprisingly, the EMCCD outperforms the CMOS in localization precision under such conditions. However, as the signal level and background level increase, these factors are less important. In the high background case shown in Fig. G.2c)-d), the CMOS is now the better camera. The main factor that gives advantage to the CMOS is the additional noise induced by the electron multiplying gain process in the EMCCD, which results in some uncertainty in the number of photons in each pixel. The CMOS also has smaller effective pixel sizes (108nm compared to the EMCCD’s 160nm), which gives some advantage when the signal level is high.
Appendix  H

Digital Counts to Photon Conversion

The first step in processing raw SMLM data is a simple conversion from digital units to photons. The conversion coefficients depend on the camera model and settings. Although conversion to photons is not strictly necessary, the segmentation and localization steps involve thresholds. Units of digital counts can vary significantly depending on camera settings, whereas photon levels are more predictable for a given dye. Furthermore, a knowledge of the number of photons is useful for predicting localization precision. A precision estimate is important for the final two steps or super-resolution image rendering and analysis.

The specific values for conversion are also different for cameras of the same model, and can also change over time. The values can be calculated experimentally. However, the calibration process must be repeated for every camera mode and gain setting combination. Camera manufacturers typically provide this information with shipment. An example of the table provided with an Andor EMCCD is shown in table H.1.

For convenience, the process of converting from digital counts to photons is combined with a function that reads raw Tagged Image Files (TIF). The function takes inputs that include camera model, serial number, and gain settings. Not shown in the table above is the effect of Electron Multiplying (EM) gain; newer models of most EMCCDs have “linear EM gain,” which means the EM gain setting is calibrated such that it is a direct multiplier. The
Table H.1: Conversion from Digital Counts to Photons for an EMCCD. This table is an abbreviated version of the system test data provided with an Andor EMCCD.

<table>
<thead>
<tr>
<th>Readout Rate</th>
<th>Preamp Setting</th>
<th>CCD sensitivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 MHz</td>
<td>1</td>
<td>14.0</td>
</tr>
<tr>
<td>17 MHz</td>
<td>2</td>
<td>8.66</td>
</tr>
<tr>
<td>17 MHz</td>
<td>3</td>
<td>4.75</td>
</tr>
<tr>
<td>10 MHz</td>
<td>1</td>
<td>15.6</td>
</tr>
<tr>
<td>10 MHz</td>
<td>2</td>
<td>7.84</td>
</tr>
<tr>
<td>10 MHz</td>
<td>3</td>
<td>4.55</td>
</tr>
<tr>
<td>5 MHz</td>
<td>1</td>
<td>17.2</td>
</tr>
<tr>
<td>5 MHz</td>
<td>2</td>
<td>8.13</td>
</tr>
<tr>
<td>5 MHz</td>
<td>3</td>
<td>4.24</td>
</tr>
<tr>
<td>1 MHz</td>
<td>1</td>
<td>17.1</td>
</tr>
<tr>
<td>1 MHz</td>
<td>2</td>
<td>7.98</td>
</tr>
<tr>
<td>1 MHz</td>
<td>3</td>
<td>4.25</td>
</tr>
</tbody>
</table>

* Electrons per A/D count.

The conversion from digital counts to photons for an EMCCD is given in Eq. H.1.

\[ N_{PE} = (DC - O) \times \frac{s}{EM} \]  

(H.1)

where \( N_{PE} \) is the number of photoelectrons, \( DC \) is the digital counts, \( O \) is the camera built-in offset (nominally 100 counts for many manufacturers), \( s \) is the sensitivity as in table H.1, and \( EM \) is the EM gain setting. The number of photo-electrons is the same as the number of detected photons; however, the number of photons striking the detector can be estimated by dividing by the wavelength-dependent quantum efficiency.
Appendix I

Super-resolution Image Rendering

The final stage in the processing of SMLM data is to present the information in a usable way. The first step is to render a super-resolution image using the (x,y) or (x,y,z) coordinates of the localizations. The standard approach is to render each localization as a 2D Gaussian function with the size corresponding to the precision of localization. The result is an image that resembles a standard fluorescence image in character, albeit with enhanced resolution. To demonstrate the utility of this visualization technique, Fig. I.1 shows a simple plotting of the coordinates of a 2D data set alongside the rendered image of the corresponding region. The image rendering is a more accurate representation of the quality of the results; the resolution of the sample sometimes appears deceptively fine when localizations are plotted as points. With 3D information, presentation of the results must be altered to make the third dimension perceptible. One technique is to encode the depth as color, as in Fig. 3.11 from Chapter 3 (see Page 58).

Super-resolution analysis does not end with the rendered image. Quantitative results require more specialized techniques that depend on what aspect of the object is of importance. In Section 3.5, I presented a measurement of the radius of a microtubule using STORM data. In that experiment, the dyes were found to be distributed on a hollow cylinder. By fitting a section of data to a line in 3D space and cataloging the distance of the localizations from the fit line, I was able to measure the physical size of the structure. Microtubules
Figure I.1: **Super-resolution Image Rendering.** The result of super-resolution analysis is a list of points, but each point also has an associated intensity. A simple plotting of the points, as in a), does not reflect the intensity information. By displaying the points as 2D Gaussian functions with a width inversely related to the intensity, the rendering in b) better conveys the full information from a STORM data set. Scale bar: 1µm.

are an interesting object to study with super-resolution, but their physical dimensions are known previously from electron microscopy [75]. Many other sub-cellular structures cannot be inspected with electron microscopy; super-resolution with optical wavelengths might be the only option for some objects. Such instances have the most potential to enable a new understanding of biological structures and processes.
Appendix J

Two-Color Double Helix

The images resulting from the 2-color DH-PSF system from Fig. 4.17 are shown in Fig. J.1. The sample consists of 100nm polystyrene beads in an imaging dish. The beads are coated with four dye species such that they can be induced to emit light in any of the four pass bands of the quad-band dichroic. The three images (a,b,c) in the figure are from the back port, the left port, and the right port. The back port sees the orange color band (581nm to 624nm), whereas the left and right side ports see red (673nm to 736nm). All three images are in focus; the arbitrary orientations of the DH lobes are due to the orientation of the masks. In the modules used here, the mask orientation depends on the threading of the camera and DH module and cannot be controlled. The most notable characteristic of the images from the back port and left port is the reduced field of view. Only $\frac{1}{4}$ of the detector is receiving light, and the quality of the DH-PSF is too poor to be usable at the edges of the reduced field. Additionally, the field is shifted from the axis. The back port (a) is not as bad as the left port (b), but both have a field too small to be useful. Interestingly, when the DH module is moved from the left port to the right port, the resulting image (shown in c) does not suffer the same field effects. The quality of the DH-PSF is somewhat reduced at the edges of the field (visible as an asymmetry in lobe intensity), but this is to be expected; the quality is still sufficient for localization.

There are several differences between the same microscope ports that might account for
Figure J.1: **Double Helix Vignetting.** These images show an in-focus scene of 100nm beads imaged with a DH-PSF through three ports of the same microscope: the back port, left side port, and right side port, shown in a), b), and c), respectively. Vignetting originates in the aberrations of the commercial microscope port and causes intolerably poor performance in the back and left ports, but the right port performs well.

The performance difference. The left port has an additional module with an astigmatic lens for 3D localization. For these experiments, the astigmatic lens was disengaged from the system, but there remains a lens system for relaying the image plane such that the astigmatic lens is in infinity space. The exact specifications of the relay system are proprietary. Furthermore, with the astigmatic lens disengaged from the system and the DH module removed, the left port was observed to have a linear phase component. When an in-focus point source is moved slightly from the focal plane, the image of the emitter on the camera shifts laterally. The same effect was observed in the back port. The back port uses a different tube lens than the two side ports. The optomechanical layout constrains the distance from the back aperture of the objective to the tube lens to be longer than the ideal distance, which is the sum of the focal lengths. Although the linear phase does not cause significant problems in any of the ports when the camera is placed at the exit ports of the microscope base, the act of relaying the image causes the aberrations to become evident. There is little to be done within the microscope base to address this problem; the optics are not meant to be adjusted by the customer. The solution, as described in Chapter 4, is to use the functioning right port for all 3D imaging. If the channels are to be split onto different cameras (or different
regions of the same camera) for multi-color 3D, the spectral filtering must occur outside of the microscope base to avoid the aberrations in the left and back ports.
Appendix K

Theoretical Localization Precision

The resolution enhancement in SMLM comes from the ability to precisely localize individual fluorescent probes. For the STORM dye Alexa 647, the emission wavelength is 670nm; the diffraction-limited image of the PSF from a 1.49 NA objective has a null-to-null width of 550nm. The precision of localization, on the other hand, can be arbitrarily high. The precision is limited mainly by the number of photons detected from the emitters. Precision of a few nanometers is experimentally achievable. Other factors that affect the localization precision are wavelength, objective NA and magnification, camera pixel size, background noise, camera QE, and camera noise factors.

A theoretical analysis of localization precision is presented in Ref. [24]. The authors consider first the case of 2D localization precision in the absence of background noise. The predicted relationship is:

\[
\langle (\Delta x)^2 \rangle = s^2 + \frac{a^2}{12N},
\]

where \(\Delta x\) is the localization error, \(\langle \rangle\) is the expectation value, \(s\) is the standard deviation of the PSF, \(N\) is the number of photons detected, and \(a\) is the size of the pixel (where \(a < s\)) [24]. This identical equation was given in Chapter 1. However, in thick samples, the effect of background is significant. Conceptually, background noise is the presence of photons that did not arise from the emitter. The effect of background can be predicted by accounting for the increased variance of the photon counts per pixel. The result of this analysis in 2D
results in an additional term to Eq. K.1:

\[ \langle (\Delta x)^2 \rangle = \frac{s^2 + a^2/12}{N} + \frac{8\pi s^4 b^2}{a^2 N^2}, \]  

(K.2)

where \( b \) is the number of photons on background noise per pixel. For the parameters of the experimental system described above, the relationship in Eq. K.1 is plotted in Fig. K.1 for a range of emitter intensities and background levels.

An alternate analysis of localization precision that enables the incorporation of accurate models of the system and noise is obtained with the Cramér-Rao Lower Bound (CRLB) [112, 113, 114]. A measurement system such as a microscope carries a finite amount of information. The CRLB provides the performance limit for an unbiased estimation algorithm that fully utilizes the information transmitted by the optical system. For examples of CRLB calculations, including several cases involving the DH-PSF, see Refs. [35, 36, 92].

Figure K.1: **Localization Precision Estimates.** Predicted localization precision is plotted over the range of experimentally realizable emitter intensities and background levels.
Appendix L

Sample Preparation of Viral Cells

The sample preparation methods for the images in Section 4.4.1 is as follows:

**Cell Lines and infections:** C57 mouse embryonic fibroblasts (MEF) were obtained from ATCC (SCRC-1008; Manassas, VA). MEFs were grown in DMEM supplemented with 10% fetal bovine calf serum (FBS) and 55μM BME. Virus strain NG59RA was used for all cell culture infections. Cell culture infections were carried out as described previously. Cells were plated on imaging dishes (MatTek, P35G-0.170-14-C) and infected at a multiplicity of infection of approximately 10 pfu/cell for 24–32 hours for STORM analysis [58].

**Immunostaining:** Dishes were washed twice with ice cold PBS, pre-extracted with 0.5% triton in CSK Buffer on ice for 5 minutes, washed 2x with ice cold PBS and fixed with 4% PFA for 10 minutes. The fixed sample was quenched with a 0.1% sodium borohydride solution to reduce nonspecific fluorescence. Following an overnight block in 10% FBS in PBS, dishes were incubated with labeled primary antibody diluted in blocking buffer at 37°C for 1hr. Samples were washed three times in PBS and post-fixed in 4% PFA for 10 minutes, then washed 2x in blocking buffer. Samples were stored at 4°C in PBS until imaging.

**Antibody labeling:** Antibodies were labeled with either ATTO 565 or Alexa Fluor 647 NHS Ester according to manufacturers protocol, with an estimated degree of labeling between 1 and 2.

**Antibodies:** Lamin B antibody (Santa Cruz, sc-6216) was used at a 1:100 dilution,
Rat anti-T-antigen antibody (E1, gift of Tom Benjamin) and Rabbit anti-VP1 antibody (I58) were used at a 1:500 dilution.

**STORM Imaging Buffer:** Imaging was carried out in STORM imaging buffer: 100mM Tris (PH 9.0), 10% Glucose, 10mM NaCl, 2% Beta Mercaptoethanol (BME) and 1% enzymatic oxygen scavenger system (Glucose oxidase (50mg/ml) and catalase (4mg/ml)) [76].