USING PHOTO-INSTABILITY TO QUANTIFY FLUOROPHORES
AND ACHIEVE SUPER-RESOLUTION IMAGING

BY

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ABSTRACT

This dissertation presents techniques for localizing and quantifying fluorophores in biological imaging applications. Fluorophore photobleaching, blinking, binding, etc., is exploited to quantify and localize single fluorophores, even when their fluorescent images overlap those of nearby fluorophores. In the first technique, we image single, membrane-bound receptors labeled with fluorophores and count the stepwise drops in fluorescence intensity to determine the number of ligand binding sites. Results from single α7 and neuromuscular junction nicotinic acetylcholine receptors in mammalian cell membranes are shown. The results indicate that there are two bungarotoxin binding sites in neuromuscular junction (NMJ) receptors, as expected, and five in α7 receptors, clarifying previous uncertainty. The other techniques are associated with super resolution imaging. Super-resolution imaging is achieved by localizing diffraction-limited spots corresponding to single fluorophores with high accuracy. In photobleaching and intermittency localization microscopy (PhILM), fluorophore transitions between dark and bright states (compatible with binding, photobleaching, photo-activation, blinking, etc.) are localized. We show that standard photobleaching and blinking movies can be used to create super-resolution images. We also explain how PhILM can be combined with another technique to image chromosomal DNA inside cells. In PAINT (point accumulation for imaging in nanoscale topography), the accumulated, stochastic binding events of fluorescent labels to an imaging target are localized. Combining PhILM and PAINT results in a robust microscopy that is faster than PAINT alone, requires less optimization, and corrects for cell autofluorescence. We used nanomolar concentrations of SYTO (which shows >40x fluorescence enhancement upon binding to DNA) to image chromosomal DNA in fixed cells. We found an average single-fluorophore localization error of 24 nm. We similarly imaged microtubules using fluorescent paclitaxel and streptavidin-based labeling to find 10 and 18 nm errors, respectively. Future work will involve simultaneous imaging of DNA, microtubules, and other proteins to answer important biological questions.
For Mom, Dad, Grandpa, and especially Grandma, who rarely lets a visit go by without bringing up the importance of education.
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This thesis represents only a small fraction of the projects on which I have actually worked during my graduate studies. Some of the other projects that are not included in this thesis have often taken significant amounts of time or have contributed significantly to my education, and since many of them will likely not be mentioned elsewhere, I will mention them here. Some of the more significant studies included a project with Enrico Gratton and William Mantulin on modifying average photon trajectories through scattering material by modifying an optical needle’s reflectance. This project resulted in a first author publication (1), a conference paper (2), and an invention disclosure to the University of Illinois. I also began work on a scanning system for optical imaging of scattering tissue using near-infrared, frequency domain spectroscopy. This project was continued by other students after the Laboratory for Fluorescence Dynamics relocated to Irvine, California. In the Selvin lab, I worked on a project to understand the asymmetric walk of the kinesin motor, which is now an ongoing project with another student, Marco Tjoe; a project to understand the rotation of the stalk domain of myosin VI, which had too many technical difficulties to continue; construction of a two-photon laser-scanning microscope; imaging single rhodamine fluorophores and quantum dots using two-photon microscopy, which is being pursued by another student, Ruobing Zhang; looking for fluorescence enhancement of labeled cells in a solution of colloidal gold, in which we could not see any enhancement (the project was then dropped); imaging single AMPA receptors labeled with a pH-sensitive green fluorescent protein for the purpose of counting of subunit ratios, which is also now pursued by another student, En Cai; and measuring distances between bound bungarotoxins in nicotinic acetylcholine receptors, which is now being further pursued principally by Hannah Deberg and Nir Friedman. The last project indirectly helped lead me to the development of PhILM, which is the subject of chapter three. In addition to these projects, I have also helped write several grant applications and research proposals, and I am first author of a book chapter on FIONA (3). I have also written invention disclosures to the University of Illinois for PhILM and transient-labeling PhILM, and at the time of submission of this thesis, the University has filed a provisional patent application for PhILM. I gained much insight into the process of acquiring patents, as much of the
application was actually written by myself, with the final version and more legal aspects written by our very helpful lawyer, Richard Osman.


Chapter 1: Introduction

Fluorescence microscopy is a powerful technique for specifically imaging virtually any part of the cell. Indeed, methods exist for labeling proteins, lipids, and nucleic acids with fluorescent molecules. In addition, a variety of specialized fluorescence microscopy techniques exist, including fluorescence lifetime imaging microscopy (FLIM), fluorescence correlation spectroscopy (FCS), and, with the advent of very sensitive detectors including avalanche photodiodes and EMCCD cameras, single-molecule imaging and tracking. These techniques allow for imaging, probing local fluorophore environments, and understanding the dynamics of labeled molecules. This thesis focuses on single-fluorophore detection within an ensemble of fluorophores. The information about individually detected fluorophores is then used to quantify fluorophores bound to cell membrane receptors and to create super-resolution images.

1.1 Fluorophore behavior

Molecular fluorescence typically involves the absorption of a photon to excite an electron from the highest occupied molecular orbital to the lowest unoccupied molecule orbital (1). The transition is to an excited singlet state. Energy is then released by vibronic or rotational transitions. Finally, a secondary photon is released that has less energy, and hence a longer wavelength, than the excitatory photon (1). Fluorescent molecules, or fluorophores, are brighter (i.e., emit more photons per unit time) when they have large absorption cross sections or have short-lived excited singlet states, which allow them to absorb and emit photons at a faster rate.

Fluorophores are inherently unstable. The occasional transition to a long-lived, non-photon-emitting “dark” state is what constitutes fluorophore “blinking.” Blinking is typically thought to involve transitions from short-lived singlet states to long-lived triplet states. The molecules eventually transition back to the ground state where they can again be excited by excitatory photons. Various strategies exist for modifying the lifetimes of the dark states (2, 3).
Fluorophores can also undergo photon-induced chemical reactions. These reactions often result in permanent dark states, and the fluorophores are said to have “photobleached.” Stronger laser excitation power results in faster photobleaching of a sample of fluorophores.

Photo-activation is the photon-induced activation of a fluorophore. It is important for performing photo-activated localization microscopy (PALM) (4) and stochastic optical reconstruction microscopy (STORM) (5), which will be discussed in chapter three.

1.2 Single-molecule techniques typically require stable fluorophores

Single molecule techniques typically require very stable, bright fluorophores. FIONA (fluorescence imaging with one-nanometer accuracy) (6) involves imaging a single fluorophore and localizing it with nanometer accuracy. FIONA is often used to track single fluorophores attached to molecular motors as they travel stepwise along a microtubule or actin filament. In these studies, identifying individual steps is often of key interest (7-10). If the fluorophores photobleach quickly, the average length of the trajectory that can be tracked, and thus the number of observed steps, is reduced. If the fluorophores blink on time scales that are similar to the single frame acquisition time of the imaging camera, then the fluorophore disappears for one or more frames, and steps might be missed. Using stably emitting fluorophores is therefore critical in many applications of FIONA.

Similarly, single-molecule Förster resonance energy transfer (FRET) experiments often require stable fluorophores. DNA Holliday junctions are structures that are important in the recombination of homologous strands of DNA. The junctions are thought to exist in a dynamic equilibrium of two states, and the lifetimes of the two states have been measured using FRET techniques (11). The “arms” of the junction can be labeled with a FRET pair of “donor” and “acceptor” fluorophores. The acceptor fluoresces when the donor is excited and the fluorophores are in close proximity to each other. When they are far apart, only the donor fluoresces. By inspecting the fluorescence-versus-time trace of the acceptor emission, transition rates can be established for the Holliday junction conformations. However, once the donor or acceptor photobleaches, no further information can be gained about the transitions. Also, if the donor or acceptor blinks, observation of the Holliday
junction transitions can be complicated. Thus, it is important to reduce blinking and in the photobleaching lifetime, or characteristic amount of time before a fluorophore photobleaches, as much as possible.

1.3 Fluorophore instability can be used to count fluorophores and perform super-resolution imaging

While in many cases it is desirable to have stable, non-blinking and non-photobleaching fluorophores, the instability of fluorophores can in fact be used to one’s advantage in several applications. For example, in fluorescence recovery after photobleaching (FRAP), the dynamics of cell membranes and transport of membrane-bound proteins can be measured by first photobleaching fluorophore-labeled lipids or proteins in small sections of cell membrane and then recording the rate of fluorescence recovery in the affected area. This can be used to understand how nearby fluorophores diffuse or are transported into the photobleached area (12).

This thesis introduces new, single-molecule based techniques that also take advantage of photobleaching. Chapter two discusses how photobleaching can be used to quantify the number of ligand binding sites in membrane-bound receptors. By using fluorescent ligands with very slow off-rates, single receptors in a cell membrane can be labeled, and the number of bound, fluorescing ligands can be counted. When expressed in a membrane at low density, labeled receptors appear as single spots on a dark background in camera images. Image sequences of the spots are recorded, and because of the stochastic photobleaching of the individual fluorophores, the intensity-versus-time trace of a single spot shows stepwise drops in intensity until all of the fluorophores are photobleached. Each stepwise drop is assumed to correspond to a photobleaching fluorophore. We used this approach to count the number of bungarotoxin binding sites in neuromuscular junction and neuronal α7 nicotinic acetylcholine receptors (nAChRs). We counted the photobleaching steps using very bright and photostable fluorescent dyes conjugated to bungarotoxin, which was important so that the photobleaching steps were well separated in time. For some of the data, we actually counted photobleaching steps while receptors were diffusing in the cell membrane, but most data were for fixed cells wherein the
receptors were immobilized in the membrane. Our data indicated that neuromuscular junction nAChRs have two binding sites, and α7 receptors have five (13). Thus, by using fluorophore photobleaching, we were able to quantify the binding sites of nAChRs in cells.

Chapter three discusses how photobleaching and blinking can be used to create super-resolution images. Image resolution refers to the minimum spacing between fluorophores at which fluorophores are still distinguishable. For visible light, diffraction predicts a resolution limit near 250 nm in most bio-imaging applications, but super-resolution imaging allows fluorophores to be much closer. Photobleaching and intermittency localization microscopy (PhILM), is introduced. While other methods exist for doing super-resolution fluorescence microscopy, PhILM demonstrates that it is unnecessary to see single fluorophores to achieve super-resolution imaging. Using a bright fluorophore and a sensitive camera, we can subtract consecutive images from each other to find the individual fluorophores that photobleach, blink, bind, photo-activate, etc., in transitioning from one frame to the next. A composite, super-resolution image can then be plotted. This analysis approach thus makes it possible to create super-resolution images from standard fluorescence imaging movies where there can be many crowded, simultaneously-fluorescing fluorophores. We applied the PhILM technique to fluorescent microtubules attached to glass, microtubules in fixed cells, axonemes, and blinking quantum dots attached to lamb receptors on E. coli. This technique works with virtually any fluorophore that is sufficiently bright and stable enough to be localized on a fluorescent background.

In chapter four, super-resolution imaging techniques are further developed, but in this case the labeling technique, not the analysis method, is the focus of attention. We combined PAINT (14) with PhILM to further expand the toolbox available to those interested in super-resolution imaging. In PAINT, the accumulated, stochastic binding events of fluorescent labels to an imaging target are localized. Fluorophores diffuse in solution and stochastically bind to the labeling target. The fluorophores subsequently blink, photobleach, or unbind. PhILM subtracts consecutive images from each other to find the individual fluorophores that bind, etc., in transitioning from one frame to the next. Combining the two techniques results in a robust microscopy that is faster than PAINT alone, requires less optimization, and corrects for cell autofluorescence. In addition,
background noise due to fluorescent labels in solution can be virtually eliminated by using labels that fluoresce only when bound to the target. We demonstrate these combined techniques using microtubules and chromosomal DNA in cells. Together, our analysis and experimental approaches simplify the experimental techniques for acquisition of data, expand the set of fluorophores that can be used, reduce the equipment requirements, and increase the potential range of imaging targets in super-resolution microscopy. These results have been submitted and are under review for publication in Nature Methods (15).

It is hoped that the methods presented here will be found to be useful by a host of investigators who are interested in understanding biology at the super-resolution level.

References for Chapter 1


Chapter 2: Using photobleaching to quantify ligand binding sites in membrane-bound receptors

In this chapter it will be shown how the stochastic photobleaching of fluorescent ligands bound to single receptors can be used to infer the number of ligand binding sites in the receptor. The photobleaching step-counting approach is not new (1), although the use of bungarotoxin to label the ligand binding sites with dye fluorophores in a cell is. We used the technique to quantify the number of α-bungarotoxin binding sites in both neuromuscular junction (NMJ) nicotinic acetylcholine receptors (nAChRs) and α7 nAChRs. Our original intent was to observe potential changes in the stoichiometry of subunits in α4β2 nAChRs associated with nicotine exposure by genetically inserting bungarotoxin binding sites into the subunits. However, we found that our implementation of the bungarotoxin labeling system caused problems that would make it impossible to make reliable observations of changes in subunit stoichiometry (in short, the insertion of a binding site in the α4 and β2 subunits changed their association properties). As there were other exciting, high-impact projects to pursue, development of an alternative labeling scheme was not pursued. Perhaps, however, this question of stoichiometry changes according to nicotine exposure will be pursued by a future graduate student.

2.1 Understanding subunit composition and ligand binding sites is useful for medicine

Membrane proteins constitute about 30% of all cellular proteins (2) and more than 40% of the targets for all commercial drugs (3). Several receptors are composed of multiple subunits, the types and stoichiometries of which are either unknown or change according to external stimuli (such as neuronal AMPA receptors under hypoxia [4]), or are expected to change (such as the aforementioned α4β2 nAChRs [5, 6]). The ability to count the number of ligand binding sites or subunits in single, membrane-bound receptors in mammalian cells, with a high signal-to-noise ratio, is therefore useful for many ion channel studies (1, 7, 8).

1 The majority of the results in this chapter have been accepted for publication in Biophysical Journal (33).
2.2 Nicotinic acetylcholine receptors (nAChRs)

nAChRs are a class of receptors that are widely studied because of their prevalence in the body and for clinical reasons. Tobacco smoking is the leading cause of preventable death in developed countries worldwide (9), and nicotine in tobacco is responsible for addiction (10). Nicotine addiction arises from nicotine binding to nAChRs in the brain (11), which likely results in a change in the stoichiometry of α4β2 nAChRs. As a result, nAChRs in general are of considerable clinical interest.

nAChRs are found both in neuron-neuron and neuromuscular junctions. These ligand-gated channels bind acetylcholine and allow ions (Na⁺, K⁺, and Ca²⁺) to flow through the cell membrane. The two major groups of acetylcholine receptors are nicotinic and muscarinic, for which nicotine and muscarine are agonists, respectively (12). nAChRs are pentameric, being composed of five similar subunits (see Figure 2.1). There are several subunit types that are grouped according to sequence homology comparisons, and the groups are α, β, γ, δ, and ε. Within each group, the individual subunit types are labeled α2, α3, α4, etc. These subunits are combined in specific ways to generate different types of nAChRs. For example, the α7 subunit forms homopentameric nAChRs. α7 nAChRs are found in the central nervous system. By comparison, the neuromuscular junction nAChRs contain two α, one β, one γ or one ε, and one δ subunit (making five total subunits). Most receptors contain α and β subunits (13-15), and α4β2 nAChRs make up over 90% of high affinity nicotine binding sites in the brain.

2.3 The number of bungarotoxin binding sites is controversial in α7 nAChRs

Some nAChRs bind extremely tightly to a protein found in the venom of Bungarus multicinctus (a southeast Asian snake). For some of these receptors, the binding constant is 94 pM, and the off-rate is on the order of a day (16). nAChRs that bind bungarotoxin include neuromuscular junction (NMJ) nAChRs, which bind bungarotoxin at two bungarotoxin binding sites (BBSs) (17, 18), and α7 nAChRs. In contrast to NMJ nAChRs, the number of BBSs and general structure of α7 nAChRs are not well characterized, although it is established that α7 nAChRs are homo-pentamers (19). A first thought would suggest that there might be five bungarotoxin binding sites in the α7 receptor because there are
five identical subunits. However, Rangwala et al. suggested that there might actually be fewer than five BBSs in the α7 receptor, based on data from alkylation of the sites using bromoACh techniques (16). They suggested that the number of BBSs might be reduced from five binding sites due to post-translational modification of the α7 subunits, causing differences in α7 subunits within the homopentamer (16). We set out to determine the actual number of bungarotoxin binding sites in the α7 receptor using the photobleaching step-counting approach with fluorescent bungarotoxin ligands. We also hoped that in doing so we might develop methods that might later be used to investigate other receptors, such as the α4β2 receptor.

2.4 Review of the photobleaching steps-counting technique

By fluoresceinly labeling and imaging single ion channels, the number of subunits (or in our case, ligand binding sites) can be determined by counting the number of photobleaching events due to the photobleaching of fluorescently labeled subunits (or ligands). The photobleaching events occur stochastically in time. It is advantageous to use a stable, bright fluorophore so that the signal-to-noise ratio of the fluorescence images is high, and the average time between photobleaching steps is at least a few image acquisition frames in length. The fluorescence-intensity-versus-time trace for a single, labeled receptor shows discrete drops in intensity over time (with noise, of course) according to the number of attached fluorophores. For example, if five fluorophores are attached to a receptor, then the intensity versus time trace will show five discrete drops in intensity (see, for example, Figure 2.7). After all fluorophores have photobleached, the intensity will be zero. Ulbrich and Isacoff used this approach to determine that NMDA receptors containing NR1 and NR3B subunits, expressed in Xenopus (frog) oocytes and labeled with fluorescent proteins (GFP or dsTomato), have a 2:2 subunit stoichiometry (1). Ji et al. extended the technique to mammalian cells to count subunits labeled with fluorescent proteins in calcium channels (8). The approach has similarly been used to count the number of labeled monomers in short amyloid oligomers (20) as well as pRNAs in the ϕ29 DNA packaging motor (21). In these two cases, however, fluorescent dyes were used instead of fluorescent proteins, and the groups were investigating in vitro protein complexes bound to glass. Das
et al. have also used the approach to count subunits labeled using Alexa 647 (a dye fluorophore) in receptors deposited on glass (22).

Using fluorescent proteins to label subunits does have advantages and also limitations. The first and foremost advantage is that all of the subunits of interest are almost certain to be labeled by inserting the DNA for the fluorescent labeling protein into the DNA sequence of the subunit. Then, whenever the subunit protein is expressed, the fluorescent protein is expressed as well, and it is covalently attached. However, the attached fluorescent proteins might be inactive for any number of reasons, including misfolding. Indeed, Ulbrich and Isacoff estimated that only 80% of their GFP was actually fluorescent when imaging NMDA receptors (1). In comparison to dye fluorophores, this percentage might be quite high. Shu et al. showed that in fact approximately only 50% of their Cy3-labeled pRNA was actually fluorescent (21). Despite the higher observed percentages of fluorescent molecules, there are a number of limitations that make fluorescent proteins an unattractive choice for single molecule experiments. These include the size of fluorescent proteins, photostability, and relatively weak fluorescence intensity in comparison to many dye fluorophores (23). Monomeric green fluorescent protein is approximately 3 nm in diameter (see Protein Data Bank, code 1GFL) and is potentially large enough to disrupt some subunit interactions. Fluorescent proteins are also quite unstable, allowing only a few tens of thousands of photons, over the course of a few seconds, to be collected before photobleaching. By comparison, a million or more photons can be collected from many fluorescent dyes over the course of seconds to minutes. Finally, using fluorescent proteins fused to subunits cannot typically be used to count unidentified ligand binding sites; instead, fluorescent ligands must be used.

2.5 Development of the technique and control experiments with NMJ nAChRs
We used fluorescent dye-bungarotoxin conjugates to label NMJ and α7 receptors and observe the stepwise photobleaching behavior. Because NMJ nAChRs are known to have two BBSs (17, 18), they are useful for verifying techniques for counting the BBSs. We incubated HEK 293 cells, transiently transfected with plasmid DNA for subunits composing NMJ nAChRs, with bungarotoxin conjugated to Alexa647. This was followed by extensive
washing to remove background fluorophores, cell fixation, and imaging using total internal reflection fluorescence (TIRF) microscopy. The cells were imaged with 633 or 637 nm TIRF laser excitation on an Olympus IX70 microscope with a 100x 1.45 NA objective, a Semrock Brightline Di01-R635-25x36 dichroic mirror, a Semrock Brightline FF01-725/150-25 emission filter, and an Andor iXon+ EMCCD camera. The standard Olympus microscope stage was replaced by a Semprex low-drift stage. An oxygen scavenging system, consisting of reagents PCA and PCD (24), was used to increase photostability of the dyes. Cells were imaged by starting image sequence acquisition and then quickly moving the microscope stage until a clearly labeled cell was found, at which point the stage was no longer moved until the cell was photobleached. Cells with labeled receptors were clearly discernible from background (see figure 2.2), except when the cells were incubated in media containing fetal bovine serum (FBS) shortly before imaging. It was found that FBS produces a strong fluorescent signal in the range where Alexa647 and Atto647N emit, and it was quite stable when PCA and PCD were added. This was reduced or eliminated by washing the cells several times and then incubating the cells in OPTI-MEM I (Invitrogen) with no added FBS prior to imaging. As a control to verify whether or not bungarotoxin was binding to receptors, we also attempted to label non-transfected HEK293 cells. We saw no labeled receptors, confirming the specificity of the bungarotoxin for the nAChRs. It was found later that we could improve our labeling efficiency by eliminating some of the washing steps. This improvement in labeling efficiency, however, did come at the cost of having more fluorophores attached non-specifically (that is, not attached to a receptor binding site) to the glass surface.

In the labeled cells that were expressing NMJ receptors, we found that many fluorescent spots showed one or two photobleaching steps, whereas very few spots showed three or more photobleaching steps (see Figure 2.6). This indicated that the NMJ receptors in fact do have two bungarotoxin binding sites, and the technique can be applied with reasonable confidence to α7 receptors. In order to count the photobleaching steps, software was used to automatically detect each spot and output its fluorescence intensity as a function of time. The numbers of photobleaching steps were generally counted by eye, and traces that did
not show clear photobleaching steps were rejected (which accounted for 30-40% of the spots).

The traces with more than two photobleaching steps were likely due to two or more nAChRs being within the radius of a diffraction-limited spot. The number of photobleaching steps could also be increased due to non-specific fluorophores found in the same spot. In an effort to eliminate some of these spots that were not composed of a single, fluorescent receptor, we rejected traces that showed abrupt shifts in position that were coincident with photobleaching steps. The center of a spot was tracked by fitting its image to a two-dimensional Gaussian of the form

\[ I(x, y) = I_0 \exp \left( -\frac{(x - x_0)^2}{2\sigma^2} - \frac{(y - y_0)^2}{2\sigma^2} \right) + B \]

where \( I_0 \) is the peak fluorescence intensity, \((x_0, y_0)\) is the center of the spot, \(\sigma\) is the spot width, and \(B\) is the background and camera offset counts. Large position shifts that are coincident with photobleaching steps can be attributed to one or more fluorophores not being associated with the same receptor. As the fluorophores photobleach, the position of the center of the fluorescent spot shifts according to the spatial distribution of the remaining fluorophores that contribute to the spot image. The diameter of a nAChR is approximately 10 nm (see Protein Data Bank, code 2bg9 [25]). We chose to reject spots that show position shifts larger than 15 nm, which is small enough to reject many spots that contain multiple receptors and/or non-specifically bound fluorophores, yet large enough to be confident that the center has really shifted and is not due to noise. For an example trace that demonstrates a rejected spot, see Figure 2.5.

2.6 Applying the technique to α7 nAChRs

To determine the number of BBSs in α7 nAChRs, we labeled the receptors with Alexa647-bungarotoxin and counted the number of BBSs using the same methods used to count BBSs in NMJ nAChRs. In actuality, we did not use the wildtype α7 subunit because α7 nAChRs are not normally transported to the cell membrane without co-expression of ric-3 (26). Instead of co-transfecting cells with ric-3 and α7 plasmid DNA, we used a chimaera of α7 and 5HT3 (serotonin receptor) that is transported to the cell surface without the presence
of ric-3 (16). Insertion of 5HT3 should not affect the bungarotoxin binding properties of
the receptor because the extracellular, N-terminal BgT-binding portion of the α7 subunit,
which contains the identical sequence to the BgT binding portion of the homologous
acetylcholine binding protein AChBP (25, 27), is included in the chimaera (16, 19). The
chimaera has also previously been shown to have indistinguishable pharmacology from the
full-length α7 for several ligands, and binds bungarotoxin (16, 19). We ourselves did not
specifically perform any tests with the wildtype receptor for comparison.

We also used bungarotoxins conjugated to Atto647N (conjugated in our lab) and
tetramethylrhodamine (Invitrogen, Cat. no. T-1175). Because of the excellent
photobleaching stability of the Atto647N conjugate (around 500,000 photons could be
collected from a single Atto647N fluorophore without deoxygenation), we found it
unnecessary to use a deoxygenating system, although employing a deoxygenating system
did improve photostability.

2.7 Data analysis

We found that, for the α7 receptors, many spots showed one, two, or three photobleaching
steps, and a smaller but significant number of spots showed four or five photobleaching
steps. An insignificant number of spots showed more than five photobleaching steps. The
results of counting photobleaching steps in α7 receptors labeled with Alexa647-
bungarotoxin are shown in Figure 2.7. We postulated that five-step photobleaching was
indicative of α7 receptors having five BBSs, although we could not formally rule out the
possibility of an additional population of receptors containing four or fewer BBSs. We fit
the histogram of photobleaching spots to a binomial distribution, assuming there were in
fact five binding sites and found the labeling efficiency to be approximately 41%. The data
were fit using least-squares fitting to

\[ N(X) = A \binom{5}{X} p^X (1 - p)^{5-X} \]

where \( N(X) \) is the number of spots showing \( X \) photobleaching steps, \( A \) is a constant, and \( p \) is
the labeling efficiency. The goodness of fit of the binomial distribution to the histogram can
be estimated using the chi-squared test (28)
where $n$ is the number of bins being fitted, $y_i$ is the number of counts in the bin, $f(x_i)$ is the expected number of counts in the bin, and $\sigma_i$ is the estimated measurement error. We assumed the error in each bin to be Poissonian; thus, the error for each bin was taken to be the square root of the number of observed counts in the bin. There should be at least five counts in the bin for a meaningful value (28), so this test was only applied to bins 1-5. The number of degrees of freedom is three since there were five bins and two fitting parameters, $A$ and $p$. The chi-squared value thus obtained was 10.8, and comparing this with a chi-squared distribution gave a $P$ value of 0.013, suggesting the fit was poor. It was observed that most of the contribution to the chi-squared value was from bin 5 (for five-step photobleaching spots). Disregarding the fifth bin and scoring only bins 1-4 gave a chi-squared value of 4.1 and a $P$ value of 0.129, which value suggests the fit can be accepted if bin 5 is neglected. The reason for the discrepancy between the number of observed and expected spots that show five photobleaching steps is not easily explained, but it could be due to underestimating the measurement error, cooperativity in ligand binding (16), insufficient rejection of spots containing multiple receptors (or non-specifically bound fluorophores), or even some slight bias in the scoring cannot be completely ruled out since the photobleaching steps were counted “by eye.” Also, pre-photobleaching might have occurred unequally among the cells, resulting in slightly different labeling efficiencies for the individual cells, which might explain the data’s deviation from a binomial distribution.

If the labeling efficiency of the receptors is high, the number of binding sites is small, and the amount of non-specifically bound fluorophores is low, it is quite straightforward to determine the number of ligand binding sites: if there are $N$ binding sites, then there are many spots that show $N$ or fewer photobleaching steps and few (ideally zero) that show $N + 1$ or more photobleaching steps. We found that our labeling efficiency was low: 41% for $\alpha 7$ receptors using in-dish labeling and < 35% when extensively washing the cells (see the “Additional methods” section below).
Unfortunately, because there was a small number of spots that showed five photobleaching steps, it was important to consider other possible models that might explain the data, such as clustered receptors or receptors and non-specific fluorophores found in the same fluorescent spot. It was especially important to know whether or not the data could be explained by a four-binding-site model. We began investigating other models by applying the same statistical methods as those used for the five-binding-site model, but we fit the histogram to a binomial distribution for four binding sites instead of five,

\[ N(X) = A \left( \frac{4}{X} \right) p^X (1 - p)^{4-X}. \]

This gave a chi-squared value of 33.4 and \( P = 3\times10^{-7} \) for bins 1-5. In this case, the fit was expected to be extremely poor because the expected number of spots showing five photobleaching steps is zero in the four-binding-site model. For fitting only bins 1-4, the chi-squared value was 9.4, and the \( P \) value was 0.009. Thus, the five-binding-site model still gave a statistically better fit when bin 5 was disregarded.

Applying the same method, assuming a six-binding-site model, gave chi-squared = 8.0 and \( P = 0.046 \) for bins 1-5. In this case, the bin that contributed most to the chi-squared value was bin 4. For bins 1-4, the values were chi-squared = 7.8 and \( P = 0.028 \). Thus, the six-binding-site model gave a slightly better statistic for scoring bins 1-5, but the five-binding-site model gave a much better statistic for scoring only bins 1-4. We still expect the five-binding-site model to be much more likely than the six-binding-site model because the receptor has only five identical subunits.

\(^2\)Since there were a significant number of spots that showed five photobleaching steps, if the four-binding-site model is still to be assumed correct, the spots showing five photobleaching steps must be explained by multiple receptors within a spot or “non-specific fluorophores” also being found within the spot (these spots not being successfully

\(^2\)Thanks go to Professor Paul Goldbart for some discussion concerning ideas in this section.
rejected by the position-shifting rejection criterion). The following derived theoretical model can be used to predict or fit the histogram of photobleaching steps when non-specific fluorophores and multiple receptors in a spot are included in the model. Here “non-specific fluorophores” refer to fluorescent bungarotoxin conjugates or other fluorophores that are bound to the surface but are not attached to any receptors. This model assumes that ligand binding to any particular binding site is independent of other binding sites, and the probability of finding a receptor or non-specifically bound fluorophore in a given area is independent of the presence of other receptors (i.e., we assume statistical independence throughout the derivation). We start by recognizing that photobleaching steps can be contributed by fluorescent bungarotoxin bound to receptors or non-specific fluorophores. We assume that non-specific fluorophores and receptors are found at low enough spatial densities to allow the probability of finding the number of non-specific fluorophores or receptors within a spot area to be Poissonian. If \( n_d \) is the density of non-specific fluorophores per unit area, \( n_r \) is the density of receptors per unit area, and \( a \) is the area of a fluorescent spot, then the expected number of non-specific fluorophores in a spot area is \( \mu_d = n_d a \), and the expected number of receptors in a spot is \( \mu_r = n_r a \). The probability of finding \( x_d \) non-specific fluorophores or \( x_r \) receptors in a spot area is then given by the Poissonian distributions

\[
P_d(x_d) = \frac{e^{-\mu_d} \mu_d^{x_d}}{x_d!} \quad \text{for} \quad x_d \in \{0, 1, 2, \ldots\}
\]

\[
P_r(x_r) = \frac{e^{-\mu_r} \mu_r^{x_r}}{x_r!} \quad \text{for} \quad x_r \in \{0, 1, 2, \ldots\}
\]

Because we believe the spacing of non-specific and receptors are independent of each other, the probability of having \( x_d \) non-specific fluorophores and \( x_r \) receptors in a spot area is

\[
P(x_r \& x_d) = P_r(x_r) P_d(x_d)
\]

The number of fluorescent molecules in a non-specific fluorophore particle is further assumed to be one. However, the number of fluorophores attached to a receptor is not necessarily one. If there are \( l \) binding sites per receptor and there are \( x_r \) receptors in a spot,
then there are \( x_r l \) binding sites in the spot. If the probability of binding to a site is independent of all the other binding sites, then the probability of \( s \) binding sites being bound to a fluorophore is given by the binomial distribution

\[
P_b(s|x_r) = \binom{x_r l}{s} p^s (1 - p)^{x_r l - s}
\]

where \( p \) is the probability that a fluorescent ligand binds to a binding site. By summing over all the possible values of \( x_r \) for a given value \( s \), the total probability of finding \( s \) fluorophores bound to binding sites in \( x_r \) receptors is then

\[
P_s(s) = \sum_{x_r=s/l}^{\infty} P_b(s|x_r)P_r(x_r)
\]

where \( s/l \) is the smallest integer greater than or equal to \( s/l \).

Let

\[
\phi = x_d + s
\]

be the total number of fluorophores within a fluorescent spot (including \( x_d \) non-specific fluorophores and \( s \) fluorophores bound to receptor ligand binding sites). The probability of finding \( \phi \) fluorophores in a single spot is then

\[
P(\phi) = \sum_{s+x_d} \sum_{x_d=0}^{\phi} P_d(x_d)P_s(s) = \sum_{x_d=0}^{\phi} P_d(x_d)P_s(\phi - x_d)
\]

Expanding \( P_s(s) \) gives the general equation for the distribution of spots showing \( \phi \) photobleaching steps:

\[
P(\phi) = \sum_{x_d=0}^{\phi} P_d(x_d) \sum_{x_r=s/l}^{\infty} P_b(\phi - x_d|x_r)P_r(x_r)
\]

Starting from this general equation, we can now begin to investigate particular cases that might explain the data in Figure 2.7. If we make the assumption that the number of non-specific fluorophores is insignificant, then \( P_d(0) = \delta_{x,0} \), and \( P(\phi) \) reduces to
If we make the further assumption that the density of receptors is sufficiently low so that the probability of finding three or more receptors within a single spot is virtually zero, then \( P(\phi) \) becomes

\[
P(\phi) = P_b(\phi|1)P_r(1) + P_b(\phi|2)P_r(2) \quad \text{for} \quad 0 \leq \phi \leq l
\]
\[
P(\phi) = P_b(\phi|2)P_r(2) \quad \text{for} \quad l < \phi \leq 2l
\]  \hspace{1cm} (2.2)

To fit the photobleaching steps histograms, the above equations can be slightly modified to

\[
N(\phi) = A(1-d)P_b(\phi|1) + (Ad)P_b(\phi|2) \quad \text{for} \quad 0 \leq \phi \leq l
\]
\[
N(\phi) = (Ad)P_b(\phi|2) \quad \text{for} \quad l < \phi \leq 2l
\]  \hspace{1cm} (2.3)

where \( A \) is a constant that reflects the total number of receptors; \( P_r(1) = (1-d) \), which reflects the portion of spots containing only one receptor; and \( P_r(2) = d \), which is the portion of spots containing two receptors. The fitting coefficients are then \( A, d, \) and \( p \), where \( p \) is the binding site labeling efficiency and is included in the \( P_b(\phi | x) \) terms.

Oftentimes, however, the amount of non-specific fluorophores is rather high. In order to fit the data, we can assume again that the probability of finding more than two or three receptors within a spot is small and can be neglected. Equations can then be derived as done above for the case of receptors with no non-specific fluorophores present. In this case, however, we let \( P_r(x) = 0 \) for \( x > 1 \). Then,

\[
P(\phi) = \sum_{x_d=0}^{\phi} P_d(x_d)P_b(\phi - x_d|0)P_r(0) + P_d(x_d)P_b(\phi - x_d|1)P_r(1)
\]

If we again consider the probability of finding two or more non-specific fluorophores within the diffraction-limited spot area to also be negligible, then the expression reduces to

\[
P(\phi) = P_d(0)P_b(\phi|0)P_r(0) + P_d(0)P_b(\phi|1)P_r(1)
+ P_d(1)P_b(\phi - 1|0)P_r(0) + P_d(1)P_b(\phi - 1|1)P_r(1)
\]
If \( \phi < 0 \) or \( \phi > x_r \), then \( P_b(\phi \mid x_r) = 0 \). In the special case when \( l = 4 \) binding sites, then the previous equation can be evaluated as

\[
\begin{align*}
P(0) &= P_d(0)P_r(0) + P_d(0)P(0|1)P_r(1) \\
P(1) &= P_d(0)P(1|1)P_r(1) + P_d(1)P_r(0) + P_d(1)P(0|1)P_r(1) \\
P(2) &= P_d(0)P(2|1)P_r(1) + P_d(1)P(1|1)P_r(1) \\
P(3) &= P_d(0)P(3|1)P_r(1) + P_d(1)P(2|1)P_r(1) \\
P(4) &= P_d(0)P(4|1)P_r(1) + P_d(1)P(3|1)P_r(1) \\
P(5) &= P_d(1)P(4|1)P_r(1)
\end{align*}
\]

\[ P(\phi > 5) = 0 \]

(2.4)

where, using the equations above,

\[
\begin{align*}
P_d(0) &= e^{-\mu_d} \\
P_d(1) &= e^{-\mu_d} \mu_d \\
P_r(0) &= e^{-\mu_r} \\
P_r(1) &= e^{-\mu_r} \mu_r \\
P(s|x_r) &= \left( \frac{4x_r}{s} \right) p^s(1 - p)^{4x_r-s}
\end{align*}
\]

In the final case, where we allow the number receptors in a spot to be \( \leq 2 \), with the possibility of \( \leq 1 \) non-specific fluorophore in the spot, the equations become

\[
\begin{align*}
P(\phi) &= P_d(0)P(\phi|0)P_r(0) \\
&\quad + P_d(0)P(\phi|1)P_r(1) \\
&\quad + P_d(0)P(\phi|2)P_r(2) \\
&\quad + P_d(1)P(\phi|0)P_r(0) \\
&\quad + P_d(1)P(\phi|1)P_r(1) \\
&\quad + P_d(1)P(\phi|2)P_r(2)
\end{align*}
\]

Importantly, for four binding sites,
where

\[ P_r(2) = e^{-\mu r} \frac{\mu_r^2}{2} \]

Again, since there are a significant number of spots that show five photobleaching steps, if the four-binding-site model is still to be assumed correct, the spots showing five photobleaching steps must be explained by multiple receptors within a spot or non-specific fluorophores also being found within the spot. We explored this possibility at the risk of beginning to over-fit the data using the equations derived above. We first attempted to fit the data to the case of multiple receptors being found within a spot. We limited our analysis to the special case of only two or fewer receptors in a spot since the probability of finding three or more should become vanishingly small, assuming receptors do not tend to cluster and cannot be rejected by the position-shift criterion. In this case, the function for the expected bin values was given in equation (2.4). Bins 1-8 were fit. Then, the suggested labeling efficiency was 48%, and 12% of the spots were expected to contain two receptors. Scoring bins 1-5 using the chi-squared test gave chi-squared = 5.4 and P = 0.07 (for two degrees of freedom). However, this fit over-predicted the number of spots showing six- and seven-step photobleaching. Scoring bins 1-6 gave chi-squared = 49.3 and P = 1x10^{-10}, which strongly suggested that this model too should be rejected.

Finally, we tested the model in which receptors could be found in the same spot as non-specifically bound fluorophores. In this case, five step photobleaching traces could be explained by a receptor with four labeled binding sites being in the same small area as a non-specific fluorophore. We restricted our analysis to the cases of one receptor in a spot,
one non-specific fluorophore in a spot, or a receptor and a non-specific fluorophore in a spot. The probability distribution for the number of steps can then be given by equation (2.5) where the terms for more than one receptor or non-specific fluorophore are considered vanishingly small and dropped. The expected number of spots is then found by multiplying the probability distribution by a constant. We fit the histogram data in Figure 2.7 to this new distribution by allowing $A$, $p$, $\mu_{ns}$ and $\mu_r$ to be fit parameters, and $\mu_{ns}$ and $\mu_r$ were constrained to values $< 0.2$. We found that the distribution fit bins 1-4 well but still predicted only 2 spots for bin five. The chi-squared value for bins 1-5 was 22.8, and $P = 2 \times 10^{-6}$ (d.o.f. = 1). Thus, we concluded that the spots showing five photobleaching steps would not be explained well by a receptor with four labeled binding sites and a non-specific fluorophore. Performing a similar fit, but allowing a maximum of two receptors and one non-specific fluorophore to be contained in a spot, gave only a somewhat better fit, with chi-squared = 7.9 and $P = 0.005$ (d.o.f. = 1). Thus, although our simple five-binding-site model did not give a very good fit, it was better than the four-binding-site model fits, including the fits involving multiple receptors and non-specific fluorophores in a spot.

2.8 Observing moving receptors

As mentioned, our technique gives a drastic improvement of signal-to-noise ratio over similar techniques that use GFP to label subunits (although by this point it should be clear that labeling efficiency appears to be somewhat to be desired). This higher signal-to-noise ratio allows us to achieve a higher spatiotemporal resolution that is necessary for tracking receptors that are diffusing in a non-fixed cell membrane, while still counting BBSs (see Figure 2.8). We found that in live cells, the labeled $\alpha_7$-5HT3 receptors had a two-dimensional diffusion coefficient of $0.1 \, \mu m^2 s^{-1}$. The diffusion coefficient was calculated by measuring the mean-squared displacement. The mean-squared displacement of the receptor as a function of time is given by

$$\langle r^2 \rangle = 4Dt$$

where $D$ is the two-dimensional diffusion coefficient of the labeled receptor and $t$ is the elapsed time. We used a tracking program written for Matlab to track the receptors (29) as well as an ImageJ plugin (30). By averaging the mean-squared displacement as a function
of time for many receptors and fitting the resulting function to a line (see Figure 2.9), we estimated the two-dimensional diffusion coefficient. In order to count the number of photobleaching steps of diffusing receptors, it was necessary to completely photobleach the receptors quickly while tracking. This is because the receptors should not cross paths, which will tend to obfuscate which receptor is which after the crossing. Also, the receptors must not travel in domains in the membrane that are far away or close enough to the coverslip surface to make a significant change in the TIR excitation power applied to the fluorophores. We used a high laser intensity (though not fluorophore-saturating) and acquired images at 0.05 s per frame. The signal-to-noise ratio was high enough to see stepwise photobleaching in a few diffusing receptors, although we were unable to make out clear steps in most of the live-cell traces. Due to this and the simplicity of analyzing non-diffusing receptors, we did most of our measurements in fixed cells.

2.9 Summary and conclusions

In conclusion, we have used fluorophore-bungarotoxin conjugates to count the number of bungarotoxin binding sites in NMJ and α7-5HT3 nAChRs. We confirmed that NMJ receptors have two bungarotoxin binding sites, and we concluded that α7-5HT3 receptors have five. We found that the high signal-to-noise ratios obtained when using fluorescent dyes allow for counting subunits, even in receptors that are diffusing in non-fixed cell membranes. We further found that by using very photostable Atto647N-bungarotoxin, we could omit oxygen scavenging systems, which could be useful for cells that are sensitive to oxygen deprivation. The use of bungarotoxin to label receptors is not limited to nAChRs with natural binding sites. Indeed, short amino acid sequences have been inserted into other proteins and shown to induce bungarotoxin binding (31). Thus, the techniques reported here should prove useful for other membrane-bound protein studies in both fixed and non-fixed cells.

Additional Methods

Labeled cell preparation for live cell experiments and 1.65 N.A. objective experiments with fixed cells. HEK 293 cells were grown to 80-100% confluency in 25 cm² cell culture flasks using DMEM (Gibco) and 10% heat inactivated fetal bovine serum (FBS) (Gibco or
Hyclone). The media was replaced with 5 mL of fresh media, and the cells were returned to the cell incubator. 25 μL of Lipofectamine 2000 (Invitrogen) was added to 500 μL of OPTI-MEM (Invitrogen). The solution was allowed to incubate for 5-15 minutes. Meanwhile, a total of 10 μg of plasmid DNA containing cDNA sequences for the subunits comprising our nAChRs was mixed with 500 μL of OPTI-MEM. To form mouse neuromuscular junction (NMJ) nAChRs, we used 4 μg of α, 2 μg of β, 2 μg of δ, and 2 μg of γ subunit plasmid DNA. To form α7-5HT3 nAChRs, we used 0.1-10 μg of α7-5HT3 and enough empty PMT4 plasmid to make the total amount of added DNA equal 10 μg. After incubating the Lipofectamine 2000 with OPTI-MEM, the DNA and Lipofectamine 2000 solutions were mixed and incubated another 20-40 minutes at room temperature (a few cells were alternatively transfected using FuGENE 6 [Roche]). The resulting solution was then added to the cell culture flask. The cells were then incubated overnight before labeling with bungarotoxin, or in a few experiments, incubated overnight with 30 nM bungarotoxin (1). Cells were washed once with PBS-HEPES-EDTA solution (PBS with 10 mM HEPES, 1 mM EDTA, pH 7.4) and then incubated with gentle rotating at room temperature for 30-60 minutes in PBS-HEPES-EDTA and 30-50 nM bungarotoxin conjugated to Alexa647 (Invitrogen), tetramethylrhodamine (Invitrogen), or Atto647N. To ensure that each bungarotoxin was conjugated to only one fluorophore, we first purified the protein using HPLC, taking only the first fluorescence peak corresponding to singly-labeled bungarotoxin and verifying the mass using mass spectrometry. To remove excess fluorophores, at the end of 45-60 minutes of incubation with bungarotoxin-fluorophore, the cells were mixed with 50 mL of PBS and centrifuged at 400 g for 2-5 minutes. The supernatant was removed, and the cells were resuspended in another 50 mL of warmed PBS and centrifuged. These centrifugation-wash steps were repeated 3-5 times. At the end of the final centrifugation step, the supernatant was removed, and the cells were resuspended in Opti-MEM® I (Gibco) with no added FBS. 2 mL of cell solution were then transferred to 35 mm glass bottom dishes and incubated at 37°C and 5% CO2 for 1.5-3 hours to allow cells to flatten out on the glass surface. At the end of the incubation period, the cells were washed once with warmed PBS or DPBS and then fixed with warmed 4% paraformaldehyde solution for 20-30 minutes before imaging. For live cell experiments, this fixation step was skipped. For fixed cells, the paraformaldehyde solution was replaced with 2 mL of PBS or DPBS solution. 20 μL of 50
nM PCD and 80 μL of 60 mg/mL PCA solutions were added as a deoxygenating system to improve photostability (24). For non-fixed cells, the PCA and PCD were added directly to the cell media solution.

*Labeled cell preparation for fixed cell experiments.* We tried using the same cell labeling method outlined above for obtaining data that would produce histograms similar to those shown in Figures 1 and 2. However, we found that the following gave a much improved labeling efficiency, at the cost of more non-specific binding of fluorophores to the coverslip surface. HEK 293 cells were seeded in 35 mm glass bottom dishes coated with fibronectin one day prior to cell transfection and were grown in DMEM with 10% FBS at 37°C and 5% CO₂. The media was replaced with 2 mL of fresh Opti-MEM® I immediately prior to transfection. Transfection was carried out using Lipofectamine 2000 (Invitrogen) per the manufacturer’s instructions. Cells expressing α7 receptors were transfected using a 1:10 ratio of α7-5HT3 plasmid and empty PMT4 plasmid. Cells expressing NMJ receptors were transfected using a 2:1:1:1 ratio of α, β, γ, and δ subunit plasmids. The cells were then incubated overnight. In the morning, the cell surface was blocked using 4 mg/mL casein and 8 mg/mL BSA in DMEM for 30 minutes at 37°C and 5% CO₂. Alexa647-bungarotoxin was then added to approximately 50 nM, and the cells were incubated at 37°C for an additional hour. The cells were then washed two times with approximately 2 mL DPBS. The cells were fixed using a solution of 4% formaldehyde and 0.5% gluteraldehyde for 25 minutes. The cells were then again washed two times with DBPS. Finally, 2 mL of DPBS with 10 uL of 5 uM PCD and 100 uL of 50 mg/mL PCA (deoxygenation reagents) were added before imaging.

*Spot detection algorithm.* We wrote the following algorithm to automatically detect spots in the image (see Figures 2.3 and 2.4):

1. Dilate the image once so that each pixel’s intensity value is replaced with the greatest intensity value of the eight nearest neighbors.
2. Find the positions of all pixels that were not affected by the dilation. These are the local maxima.
3. Determine the average pixel intensity of all pixels within the diffraction limited spot radius \( R \) (generally \( R \) is chosen to be three pixels) of a given local maximum and call it the "spot average pixel intensity."

4. Determine the average pixel intensity of all pixels between \( R \) and \( R + dR \) (\( dR \) is generally chosen to be one pixel) around the local maximum and call it the "average local background intensity."

5. Subtract the local background intensity from the spot average pixel intensity. Call it the "background-subtracted spot intensity."

6. Compare this with a chosen threshold value that is well above noise. Local maxima with values that are above the chosen threshold are considered "real" spots.

7. Measure the distances between all pairs of spots. Throw away all spots that are too close together (generally, this distance is chosen to be seven pixels). Throw away all spots that are too close to the image edges (this distance is chosen to be half of the minimum separation distance between spots).

8. An output TIFF file is produced that can be compared with the original image file to check spot detection performance.

**Calculating fluorescence photobleaching traces.** We rejected movie files for which the average density of spots in the cell was \( > 0.014 \) spots/pixel, where the length of a pixel corresponded to 106.67 nm. For fixed cells, the first 5 frames in the image file were averaged, and then our spot detection algorithm was employed to find the spots in the resulting averaged image. The coordinates of the detected spot centers were used to calculate the spot fluorescence intensities in all frames. The spot intensity in each frame was obtained by repeating steps 3-5 of our spot detection algorithm. For diffusing receptors, we used tracking programs (Matlab particle tracking code from http://physics.georgetown.edu/matlab/ or an ImageJ plugin described in [30]) to identify the spot coordinates in each frame. We then again used step 3 or steps 3-5 of our spot detection algorithm to calculate the fluorescence in each frame using the coordinates obtained by tracking.

**Investigating whether position-shift rejection biases results.** From Figure 2.10, it appears that including spots that do show position shifts that are coincident with photobleaching
steps does not make a very significant difference in the final histogram. The next question, which is much more difficult to answer, is, have we introduced a systematic bias by rejecting spots based on position shifts? Ideally, all spots containing two (or more) receptors will be rejected with equal probability, in order to ensure that there is no bias toward keeping, for example, two-step photobleaching traces over keeping five-step photobleaching traces. However, there is a fear that this method will reject a higher percentage of the spots with 2-4 photobleaching steps and two receptors than it will for five photobleaching steps and two receptors. This fear arises because there is a possibility that a brighter receptor (say, for example, containing four fluorophores) might mask position shifts due to the photobleaching of the paired, dimmer receptor (with, say, one fluorophore) if the dimmer receptor photobleaches early. To get an idea of what the rejection frequencies are, the Figure 2.11 shows the rejection rates as percents of the number of spots showing the given number of photobleaching steps (they are plotted on top of the steps histogram for reference). For example, 6% of the traces showing two photobleaching steps were rejected based on position shifts.

Most of the spots are rejected for >5 photobleaching steps. This is good, considering that spots showing more than five photobleaching steps are assumed to contain two or more receptors (notice that for a four-binding-site model, we expect the majority of traces showing five photobleaching steps to also be rejected). However, what does the graph indicate for ≤5 photobleaching steps? To answer this question, we must come up with a theory of what the graph should look like if there is no bias, and hope that we have enough signal-to-noise in our graph for good comparison. To this end, we used fit values from binomial fits of the photobleaching steps histogram (not rejecting spots based on position shifts) using (2.3) to generate a graph (Figure 2.12) of the predicted percents of the spots observed that have two labeled receptors. Figure 2.12 suggests that, given this alternative fit, most of the five-step photobleaching traces are appropriately rejected. However, it also shows that many more are rejected for 2-4 photobleaching steps than would be expected. This is exactly the opposite result of our initial fear that more spots would be rejected that showed 2-4 photobleaching steps than those that show five.
This could be due to a poor binomial fit. It could also be real and the result of having non-specific fluorophores on the surface that show only one photobleaching step. The first hypothesis is not easily tested, but to test this second hypothesis, we increased the probability that a “receptor” has only one photobleaching step until the predicted rejection frequencies resembled the real rejection frequencies (see Figure 2.13). We found that increasing the probability by a factor of three improved the comparison with the observed frequencies, demonstrated in Figure 2.13. Increasing the probability by a factor of three seems like a large increase, but in fact it might not be since many dim fluorophores with one photobleaching step might not be detected due to the threshold intensity that was chosen for detecting spots.

Rejecting spots based on position shifts that are coincident with photobleaching steps seems like a very reasonable way to try to discriminate spots that contain two or more receptors (or a receptor plus a non-specific fluorophore). As seen in Figure 2.10, most of the spots showing >5 photobleaching steps that might have otherwise been counted were rejected in this way. Comparing the rejection frequencies as a function of number of photobleaching steps with a simple theoretical distribution did not show good qualitative agreement until the possibility of non-specific fluorophores was added. This addition of the non-specific fluorophores component apparently masks any underlying bias that might have been introduced by the rejection of spots based on position shifts. Thus, it appears that using the current data set, I cannot reliably measure any bias introduced by rejecting spots based on position shifts.

Regardless of whether there is a small bias or not, does the benefit of rejecting spots based on position shifts outweigh the bias we might introduce? If our goal is to see a significant drop in the number of spots showing >5 photobleaching steps (or >4 if the receptor has only four binding sites), then the answer appears to be “yes” because the method was shown to reject most of the spots that are definitely expected to contain two or more receptors (Figure 2.11). If we are worried about having a perfect binomial distribution, then the answer might be “no” because of the possible misshaping of distribution due to bias. Even in this case, though, the bias is likely to be small since two low probability criteria have to be simultaneously met:
1. The receptors must be very close together.
2. The receptor with fewer fluorophores must photobleach quickly and completely before the brighter receptor photobleaches.

We thus concluded that the cost of bias was small, if present, compared to the benefits of using the position-shift rejection criterion.
Figure 2.1. Acetylcholine receptor (AChR) from electric torpedo ray. This AChR contains 4 subunit types (each colored differently) and is very similar to human nAChRs. Crystal structure is PDB code 2bg9 (25) inserted into a lipid bilayer. Figure was rendered using VMD (32).
Figure 2.2. Example image of nAChRs labeled with Alexa647-bungarotoxin on the surface of a HEK 293 cell.
Figure 2.3. Example images demonstrating intermediate steps in the spot detection algorithm. The first five frames in the image sequence are averaged. The resulting image is dilated, and the positions of local maxima are identified because pixel intensities of local maxima do not change under dilation. The spots are then identified based on shape and intensity, using the positions of the local maxima as starting points. Finally, spots that are too close together are rejected (“thinned”).
Figure 2.4. Example use of spot detection algorithm. The spots are detected (top left, highlighted in cyan) and thinned to seven pixels minimum separation between spot centers (top right, highlighted in red). Ambiguous photobleaching traces are rejected, resulting in using only the spots highlighted in magenta (bottom) for the tallies of number of photobleaching steps.
Figure 2.5. Example of a spot (shown in the upper right corner) rejected for position shifts coincident with photobleaching steps. The fluorescence intensity of the spot is plotted in blue while the distance the spot has moved from its starting position is plotted in green. In this example, significant, sudden position shifts occur near frames 30 and 510 (other movement, especially near the beginning of the trace, can be attributed to stage drift). Here, the length of a pixel is 106.67 nm. It is also noticed that our step-fitting program was not perfect in finding all of the steps, and hence the need to ultimately count the steps “by eye.”
Figure 2.6. Histogram of photobleaching steps for NMJ type nAChRs. The histogram suggests that there are indeed only two BBSs in this receptor because most spots show two or fewer photobleaching steps.
Figure 2.7. (top) Photobleaching trace of α7-5HT3 receptor labeled with bungarotoxin-Alexa647. (bottom) Histogram of number of photobleaching steps per labeled receptor and binomial distribution fit, assuming five bungarotoxin binding sites.
Figure 2.8. Counting photobleaching steps of an α7-5HT3 nAChR in vivo. In the photobleaching trace (top), the labeled receptor appears to have five attached fluorophores: one fluorophore photobleaches near 0.15 s, two photobleach simultaneously near 1.25 s, a fourth photobleaches near 2 s, and the last photobleaches near 2.3 s. Because of diffusion, the receptor had to be tracked (shown in bottom image sequence).
Figure 2.9. Mean-squared displacement as a function of time for diffusing α7-5HT3 nAChRs labeled with bungarotoxin-tetramethylrhodamine. Fitting the function to a line allowed the two-dimensional diffusion coefficient to be estimated as 0.1 $\mu$m$^2$s$^{-1}$.
Figure 2.10. Effect of not rejecting spots based on position shifts. The overall shape of the histogram changes little when spot rejection based on position shifts is not used.
Figure 2.11. Graph showing what percent of spots are rejected for position shifts (blue) plotted on top of data histogram (red).
Figure 2.12.
Comparison of rejecting spots based on position shifts with portion of spots predicted to contain two receptors

Figure 2.13.
References for Chapter 2


Chapter 3: Super-resolution imaging using photobleaching and intermittency localization microscopy (PhILM)

Standard fluorescence images have an intrinsic resolution limit that is determined by the diffraction limit of light. The width of a spot due to a single fluorophore is typically given as $\lambda/2NA$ where $\lambda$ is the wavelength of the emitted light and NA is the numerical aperture of the microscope objective. As the typical fluorescence emission wavelength used in biological imaging is between 500 and 800 nm, the width of a spot is usually limited to >200 nm. Fluorophores that are closer together than 200 nm are thus impossible to resolve using standard fluorescence imaging because their fluorescent spot images overlap.

Special fluorescence imaging techniques have recently been developed to overcome the diffraction limit of light. Using highly sensitive CCD cameras, single fluorescent molecules can be detected and imaged as fluorescent spots. When fluorophores are spatially distributed at low density (that is, the resulting fluorescent spot images of single fluorophores do not overlap and are easily distinguishable from each other) the individual spots can be fit to two-dimensional Gaussian functions, and their centers can thus be localized with accuracy much better than 200 nm, or “super-resolution” accuracy (1, 2).

Individual fluorophores can also be imaged as single spots and localized to high accuracy, even when fluorophore density is high. Several techniques now exist to accomplish this. FPALM (3), PALM (4), and STORM (5, 6) use “photoactivatable” or “photoswitchable” fluorophores (7, 8). FPALM, PALM, and STORM are virtually the same technique, but FPALM and PALM use fluorescent proteins while STORM uses fluorescent dyes. Initially, the fluorophores are not fluorescent (or are made to not be fluorescent). Small subpopulations of photoactivatable fluorophores are then activated using short bursts of laser excitation. The laser used to activate the fluorophores emits at a wavelength that is

1 Most of the results of this chapter have been submitted to Nature Methods for publication (28).
different from that normally used for exciting the fluorophores to emit fluorescence light. This small subpopulation of fluorophores is then excited with a second laser, causing them to fluoresce and allowing their centers to be localized, until they eventually photobleach. The process is repeated many times until a large number of fluorophores have been localized. The localizations of all the fluorophores can then be plotted in a composite image that shows their arrangement with super-resolution accuracy. See Figure 3.1 for a pictorial example of the PALM technique.

More recent techniques solve the problem of simultaneously fluorescing, crowded fluorophores by forcing fluorophores into long-lived dark states associated with blinking. In this case, the same laser that causes the fluorophores to fluoresce also causes the fluorophores to blink, eliminating the need for two lasers and laser cycling. Chemical reagents are generally added that increase the lifetime of the blinking dark state. dSTORM drives fluorophores into the triplet dark state using relatively high power lasers and special photostabilizing reagents (9). In a similar way, Blink Microscopy also uses chemical reagents to extend the lifetime of the dark state [10]).

Current methods for doing super-resolution fluorescence microscopy are rather complex for the typical laboratory to apply. For example, STORM and PALM rely upon using photoactivatable fluorophores, which severely limits the set of fluorophores that can be used. Photoactivatable fluorophores are cycled on and off repeatedly using special laser cycling equipment. This requires two separate lasers: one to cause the fluorophores to activate, and a second to cause the activated fluorophores to actually fluoresce and eventually photobleach. Additional shuttering equipment is necessary for cycling the lasers. This equipment is not found in most biology labs. dSTORM removes some of the restrictions by expanding the set of fluorophores that can be used and removing the necessity of laser cycling equipment, but necessary reagents and laser intensities can be difficult to use or optimize. Among the less related super resolution techniques, structured illumination microscopy (which is not necessarily a fluorescence technique) also requires specialized equipment and is generally limited to only a two-fold increase in resolution (11, 12). Stimulated emission depletion microscopy, or STED, is a powerful technique which does not require the use of single molecules and can achieve super-resolution imaging (13),
but it does require high laser excitation powers that can cause significant photodamage to biological samples (6).

We desired to create a method that was less restrictive and more accessible to many biologists. In this chapter, it is shown that it is in fact unnecessary to see single spots to achieve super-resolution imaging. Fluorophores stochastically photobleach when exposed to laser excitation. Resulting quantized drops in fluorescence intensity can be localized, even with background from nearby fluorophores, to create super-resolution images from standard photobleaching movies. This is achieved by subtracting post-photobleaching images from pre-photobleaching images to create a difference image. The difference image shows a spot where a photobleaching event occurred. In addition, we use frame averaging and weighted two-dimensional Gaussian fitting to reduce the effects of shot noise that are inherent in the higher fluorescent background. In an analogous way, we can also localize fluorophores that blink, transitioning from dark to bright states, and vice versa. In essence then, by simply recording a movie of the photobleaching (or blinking) fluorescent image using a sensitive CCD camera, the movie can be processed offline to produce a super resolution image. This technique presents a robust way to analyze image sequences and create super-resolution images. While the other methods will ultimately yield higher resolution images, this technique can be immediately available to a much broader range of users. Furthermore, the algorithms presented here can be applied to data collected using STORM, Blink Microscopy, etc., and simplifies the problem of identifying spots that actually contain multiple fluorophores. As blinking and transient labeling (see next chapter) techniques become more highly developed and reach common use, it is in fact likely that the algorithms presented here will become primarily used for robustly analyzing data taken using those methods and not necessarily using simple photobleaching and blinking of crowded fluorophores. Alternatively, the method might commonly be applied to an intermediate of the two, which is represented by some of the data in Chapter 4. We call our technique “photobleaching and intermittency localization microscopy,” or PhILM for short.
3.1 Description of the PhILM algorithm

Figure 3.2 outlines the technique as it is applied to a simple two-fluorophore photobleaching movie. Localizing small groups of fluorophores within a diffraction-limited spot is not new and has been done before using such techniques as SHRImP (14, 15) and NALMS (16). These techniques have been effective at localizing small groups of two to five fluorophores. However, because we wish to identify the times and positions of hundreds or thousands of individual photobleaching and blinking events on a potentially complex fluorescent background, we need a systematic, automated way to do this. This can be done by creating what we call a "backwards-subtracted movie" and localizing the resulting light and dark spots that correspond to photobleaching and blinking events. The method could also have been done in a forwards-subtracted way, but backwards-subtracted is more intuitive for thinking about photobleaching. For frames labeled by index \( k \), the backwards-subtracted movie is created by subtracting frame \( (k + 1) \) from frame \( k \) to get new frame \( k' \), which is then inserted into the stack of images in the new "backwards-subtracted movie." The new frame, \( k' \), contains light and dark spots on a flat background (see Figure 3.3). Light spots correspond to photobleaching events, i.e., there is a fluorescing molecule in frame \( k \) that is not fluorescing in frame \( (k + 1) \). Dark spots indicate transition-to-bright-state events as occurs in blinking, i.e., there is a molecule that is fluorescing in frame \( (k + 1) \) but not in frame \( k \). We use a spot detection algorithm, which is similar to the one found in Chapter 2 and is described in the “Additional Methods” section of this chapter, to automatically locate the dark and light spots in every frame in the backwards-subtracted movie. After doing so, the located spots can be fit to two-dimensional Gaussian functions (or similar fitting procedures—see for example [17]) directly and used to plot a super-resolution image.

While PALM, STORM, dSTORM, etc., localize fluorophores with essentially dark backgrounds (because nearby fluorophores are not fluorescing), the same does not have to be true for PhILM. In this case, noise is governed by (1) the dark noise due to the camera in the pre-photobleaching image, (2) the shot noise in the pre-photobleaching image, (3) the shot noise in the post-photobleaching image, (4) the dark noise in the post-photobleaching image, and (5) intrinsic fluctuations in the amount of fluorescence from the fluorophores.
themselves. Thus, having many fluorophores in the background will reduce the signal-to-noise ratio. Because one frame is subtracted from another, the standard deviations of the individual pixel intensities add in quadrature to give the total pixel noise of the resulting subtracted image (18). To improve signal-to-noise ratios, the information about the times and locations of the photobleaching and blinking events are used to determine over what range, before and after events occur, that frames can be averaged. This reduces noise that might otherwise be more significant with no frame averaging. The frames are averaged until a frame is encountered in which a photobleaching or blinking event occurs that is within a given radius of the position of the event of interest. This minimum separation distance is selected by the user (we generally choose five to six pixels as the minimum distance between events). If several pre-photobleaching (or post-photobleaching) frames can be averaged to produce the averaged pre-photobleaching image (or averaged post-photobleaching image), the noise is reduced by a factor of \( \sqrt{N} \) where \( N \) is the number of frames averaged. Thus, it is advantageous, in terms of noise, to average as many of the pre- or post-photobleaching frames as possible. The total noise associated with a single pixel in the final (subtracted) image can be estimated by

\[
\sigma = \sqrt{\frac{\sigma_{A,\text{shot}}^2}{N_A} + \frac{\sigma_{A,\text{dark}}^2}{N_A} + \frac{\sigma_{B,\text{shot}}^2}{N_B} + \frac{\sigma_{B,\text{dark}}^2}{N_B}}
\]

where \( \sigma_{A,\text{shot}} \) is the shot noise associated with the pixel in the frames after photobleaching, \( \sigma_{A,\text{dark}} \) is the associated dark noise in the frames after photobleaching, and \( N_A \) is the number of frames after the photobleaching event that are averaged. By replacing the \( A \) with \( B \) in these terms, the corresponding values are represented for the frames before photobleaching. In our software, we estimate \( \sigma_{A,\text{shot}} \) as the square root of the pixel intensity (after subtracting the camera baseline counts) of the frame-averaged image. \( \sigma_{A,\text{dark}} \) is estimated by selecting a portion of the image in which there are no fluorophores present and finding the standard deviation of the pixel intensities. \( \sigma_{A,\text{dark}} \) is assumed to be equal to \( \sigma_{B,\text{dark}} \). This estimate of noise using frame averaging assumes that there is very little stage drift during the time that the averaged frames are acquired. If there is significant stage drift, the number of frames that are averaged can be restricted.
Once the averaged and subtracted image is calculated, the resulting spot image is fit to a two-dimensional Gaussian to localize the fluorophore. The Gaussian fit is done by fitting all pixels within a given radius of the brightest pixel of the spot using Levenberg-Marquardt fitting (using the GNU Scientific Library), although there are other methods to localize the fluorophore using the spot image (1, 17). For simulated fluorescent spot data with a spot width of 3 pixels and 700 counts peak intensity on a flat background (with added noise), the spot fitting accuracy did not increase significantly past a fitting radius of 3 pixels. For fitting the real data, we therefore used a fitting radius of four pixels for the Gaussian fitting. The individual pixels are weighted according to the estimated noise associated with each pixel. Using a weighted Gaussian fit reduced fit error by about 10% for the simulated single spots on dark, uniform backgrounds. It was therefore concluded that the improvement in fitting accuracy would be improved by at least 10% for a non-uniform background. The fitting accuracy is reported as the error reported by the fitting program using the covariance matrix of the best fit parameters. For simulated spots on dark backgrounds, the errors were found to be very near the fitting accuracy predicted by Thompson et al. (1) for single spots on flat backgrounds. We do not estimate the error using the Thompson et al. equation but, rather, use the error reported by the fitting program, since the Thompson et al. error equation is derived by assuming a flat background with Gaussian noise. Using the error reported using the fitting program gives the added benefit that we do not have to know the counts-to-photons conversion factor, which is required by the Thompson et al. equation, in order to calculate the localization error.

If an individual spot is not fit well by a Gaussian function, or the localization error is too large, the spot is rejected and not used to create the final super-resolution image. If two events occur in the same frame and are closer than the minimum separation distance, both events are rejected and not used in creating the super-resolution image, with the conclusion that both spots cannot be well fit by Gaussians because they are too close together. If, however, the events are very close together such that their combined fluorescence image appears as a single spot according to all of the rejection criteria, and photobleach simultaneously, the program unfortunately cannot distinguish the two events and instead recognizes them as a single event.
3.2 Equipment and display considerations

A typical microscope setup that can be used for single molecule data acquisition will include a 60-100x, high numerical aperture objective (NA ≥ 1.4); 1.5x internal microscope magnification; and a sensitive EMCCD array camera. The width of a single pixel in the fluorescence image will correspond to the real, physical widths of the camera pixels divided by the total magnification of the sample. The size of the image pixel should be approximately half the width of the fluorescent spot image of a single fluorophore (1). For example, for the experiments in this chapter, we used either an Olympus IX70 or IX71 microscope with a 1.45 NA 100x objective and an additional 1.5x or 1.6x internal lens (making the total magnification 150x or 160x, respectively). We used an Andor iXon or iXon+ camera with pixel width 16 um. This gives an effective image pixel size of 106.7 nm with 150x optical magnification or 100 nm for 160x magnification.

For PhoLM super-resolution images, the localization error of a single fluorophore is usually on the order of 10-30 nm, which makes it clear that using a pixel size of 106.67 nm will not represent well the true resolution. Instead, pixel widths in the final super-resolution images are chosen to be on the order of 10 nm (although we can choose any value we want). The localized fluorophores are then plotted as two-dimensional Gaussian functions with widths corresponding to the localization accuracy of the fit. We simultaneously plot images in which individual spot localizations are plotted as single pixels. These images, however, lack information about how well the individual spots are actually localized and their relative brightnesses.

Prior to acquiring data, it is useful to try to optimize the laser intensity and camera settings. Saturated pixels are to be avoided; however, making full use of the dynamic range of the camera is important in order to maximize signal-to-noise ratios. For EMCCD cameras, the gain should be set high enough to clearly see individual fluorophores. Frame acquisition should be fast enough to be able to separate photobleaching and blinking events in time. As the density of fluorophores decreases and the time interval between photobleaching events increases, the frame acquisition time can be increased.
3.3 Applications

3.3.1 Microtubules on glass

As an initial test of the improvement in resolution that can be achieved, we recorded movies of tetramethylrhodamine-labeled microtubules in vitro as they photobleached and then applied the PhILM algorithm to the resulting image sequences. Microtubules have a diameter of 25 nm (5). However, the apparent width of a microtubule using conventional fluorescence microscopy is governed by the diffraction limit of light, making the apparent width closer to 300 nm.

The starting labeling density was approximately 4:1 unlabeled:labeled tubulin monomers (Cytoskeleton, Cat. No. TL238 and TL590M). Microtubules were attached to a coverslip coated with a truncated form of kinesin. The microtubules were excited using 532 nm laser excitation and total internal reflection fluorescence (TIRF) microscopy. The microtubules were imaged in a solution containing beta-mercaptoethanol (Fluka) and oxygen scavenging reagents (PCA and PCD) to improve fluorophore stability (19, 20). For PhILM algorithm parameters, we chose a minimum distance of six pixels between photobleaching events for frame averaging purposes.

We found the resulting images (see Figure 3.4) much highly resolved than the standard fluorescence images. The average spot localization error—that is, the average error associated with localizing the center of an individual fluorophore—was ~20 nm. However, to further quantify the improvement in resolution, we selected short, straight microtubule segments and fit the localized spots or fluorescence intensity along the length of the microtubules to a straight line. The distribution of spots or fluorescence perpendicular to the fit line was used to calculate the widths of the microtubules in the regular and super-resolution images (see Figure 3.7). We found less than 60 nm width for TMR-labeled microtubules in the PhILM image, which is very close to what we expect, given that the diameter of the microtubule is 24 nm and the average localization error of single spots was ±20 nm. 30% of the localized fluorophore events were in fact due to transitions to bright states, suggesting that fluorophore blinking also played an important role in creating the super-resolution image.
3.3.2 Axonemes

Finding an improvement in resolution using microtubules is useful, but it is also instructive to know whether or not the technique can distinguish structures with different widths. Axonemes typically have a “9 + 2” arrangement where the circumference of the axoneme has nine doublets of microtubules running along the length of the axoneme, and a pair of microtubules running up the center of the axoneme. The diameter of isolated axonemes is expected to be between 160 and 200 nm, depending on the presence of Mg$^{2+}$ in the surrounding buffer (21). This makes the diameter at least six times greater than that of microtubules. We therefore decided to investigate whether or not we could see an increase in diameter using PhILM.

Because of the large diameter and the short penetration depth of excitation light into samples using TIRF microscopy, it was necessary to image axonemes using epifluorescence instead of TIRF microscopy. We labeled the axonemes by directly conjugating them to tetramethylrhodamine (see “Additional Methods” below). We attached the axonemes to the coverslip surface via the same method used for attaching tetramethylrhodamine-microtubules. We found a full-width-at-half-maximum (FWHM) value of 180 nm for the axonemes (see Figure 3.10), which is much larger than the value for microtubules and close to the expected value of the real axoneme diameter (160-200 nm). Thus, our super-resolution results corresponded well with the expected real diameter of the axonemes and showed that we were indeed able to see a size difference between microtubules and axonemes.

3.3.3 Microtubules in cells

We also imaged photobleaching microtubules labeled with indirect antibody staining in fixed COS-7 cells. We imaged using secondary antibodies conjugated to TMR, Alexa Fluor 647, and CF633. The regular TIR images gave microtubule widths near 500 nm (antibody-

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2 Thanks go to Vladimir Gelfand for useful suggestions on how to label microtubules using antibody staining.
labeling increases the width, and microtubules were not fit as well to straight lines in this case). In all three cases, we found significant improvement in resolving microtubules using PhILM. We found our best results using CF633 (Figures 3.11 and 3.12), followed by TMR (Figure 3.13). This is likely due to the excellent photostability and brightness of the CF633 dye. Again, we found a significant portion (often >50%) of the fluorophore localization was due to blinking. An example image can be found in Figure 3 where the average localization error of plotted, single spots was 21 nm. The average microtubule width in the PhILM image was found to be 100 nm (Figure 3.14).

We also made an attempt to image microtubules in cells by transfecting cells with plasmid pEGFP-Tub (BD Biosciences, Cat. no. 632349) for EGFP-tubulin. EGFP is a variant of green fluorescent protein (GFP), and is one of the most commonly used fluorescent proteins. Unfortunately, EGFP was apparently not bright enough or stable enough to use with PhILM, although we did have some limited success in improving resolution of EGFP-labeled microtubules attached to glass (see Figure 3.8).

### 3.3.4 LamB receptors using blinking quantum dots

Finally, we tried applying PhILM to blinking quantum dots bound to LamB receptors on the surface of *E. coli*. We were interested in knowing whether quantum dots, which blink but do not photobleach, could be used in conjunction with PhILM. The LamB receptors were labeled with biotin on the surface of live *E. coli*. Streptavidin-conjugated quantum dots were then allowed to bind, and movies were taken. The blinking of the quantum dots occurred on millisecond time scales, which required a frame acquisition time of 3 ms in order to see blinking transitions. Fortunately, quantum dots are very bright, which made it possible to collect enough photons, during the frame acquisition time, to allow localization. Individual spot localizations resulted in an average localization error of 18 nm. Results for a single bacterium are shown in Figure 3.15. Because the cells are not fixed, and because the single frame acquisition time is small, we had the unique opportunity to localize receptors as they potentially diffuse short distances through the membrane. To get a sense

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3 Thanks go to Eli Rothenberg for providing data on the LamB receptors.
of the paths that might be traced out by the receptors, we plotted the super-resolution image in much the same way images are created using a “Z Projection” using maximum pixel intensities for a stack of image frames. In our case, however, the spots were plotted one at a time, and the image pixels were only updated if the pixel intensity would be brighter after plotting the spot. For example, if a spot were to be plotted where a spot had not previously been plotted, then the image would be updated. However, if a spot were to be plotted in the same position as a previously plotted spot with the same intensity and localization accuracy, then the image would not be updated. The plot thus shows where the spots are localized, but not necessarily where they are concentrated. See Figure 3.15 for this “Z projection, maximum intensity” super-resolution image.

3.4 Theoretical limitations

The method is limited by signal to noise ratios but also by the maximum density of fluorophores that can be permitted. There are basically three ways in which the density of fluorophores limits the technique.

First, more fluorophores means that there will be more background fluorescence. This will reduce signal-to-noise and hence the fitting accuracy of the individual spots. As a simple way to get a sense of the effect of added background fluorescence, we will temporarily assume that the background is flat and make use of the results of Thompson et al. for estimating the localization error (1, 2),

$$\sigma_i = \sqrt{\frac{s^2 + a^2/12}{N} + \frac{8\pi s^4 b^2}{a^2 N^2}}$$ (3.2)

where $\sigma_i$ is the estimated localization error along the x or y direction; $s$ is half the spot width along the given direction; $a$ is the length of the pixel; $N$ is the number of photons collected from the fluorophore in a single frame; and $b$ is the background noise per pixel, in terms of photons. Let us suppose that we have $m$ fluorophores distributed randomly in an area $A = s^2$, which is roughly the size of the area of a diffraction-limited spot, and each fluorophore emits $N$ fluorophores per movie frame. Let us further assume that there are enough fluorophores to make the fluorescence intensity roughly constant within the area $A$, and the background photon counts due to dark currents, etc., are negligible compared to
the photons collected from the fluorophores. In this case, assuming the noise associated with detecting photons is Poissonian, \( b \) is approximately

\[
b = \frac{\sqrt{mN}}{\sqrt{A/a}} = \frac{\sqrt{mN}}{s/a} = \frac{a\sqrt{mN}}{s}
\]  

(3.3)

Now let us suppose that a single fluorophore photobleaches in the transition from one frame to the next. What is the estimated accuracy that can be achieved if we apply PhILM and do no frame averaging? The number of fluorophores left after the photobleaching event that will contribute to the background fluorescence is now \((m - 1)\). However, the shot noise due to the background fluorophores doubles since the spot image we fit to a Gaussian is actually the difference of two frames, the background noise in both frames should be equivalent, and the background noise due to each frame adds in quadrature (see equation [3.1]). Using our expression for \( b \), the localization error can then be estimated as

\[
\sigma_i = \sqrt{\frac{s^2 + a^2/12}{N} + \frac{4\pi s^2(m - 1)}{N}}
\]  

(3.4)

Thus, if \( m = 10, s = 125 \text{ nm}, a = 100 \text{ nm}, \) and \( N = 10,000 \), then \( \sigma_i = 13 \text{ nm} \). It should be noted that in (3.4), the last term in the square root dominates even for small values of \( m \) (i.e., \( m > 1 \)), so the localization error can be expected to increase as the square root of \((m - 1)\). It should also be noted that this equation does not account for fluctuations in \( N \) from individual fluorophores, which will further increase the localization error. Of course, localization accuracies can be improved if frames can be averaged (see equation [3.1]). If \( n \) is the number of frames that can be averaged before and after the photobleaching event, then (3.4) decreases to

\[
\sigma_i = \sqrt{\frac{s^2 + a^2/12}{N} + \frac{4\pi s^2(m - 1)}{nN}}
\]  

(3.5)

For theoretical localization accuracies for other values of nearby spots and frames averaged, see Figure 3.17.

Second, if two (or more) fluorophores simultaneously photobleach (that is, on the order of the frame acquisition time of the camera), and if these fluorophores are close enough in
space such that their point spread functions cannot be separately fit by Gaussian functions, then the two photobleaching events cannot be used to construct the final super resolution image. Therefore, the useful density of fluorophores is limited by the photobleaching rate of the individual fluorophores. The theoretical maximum density, based on these criteria, then requires that the average time between photobleaching events is long enough to fit one fluorophore per fitting area per frame.

Finally, the fluorophore density is limited by the dynamic range of the CCD camera. All CCD array cameras have a maximum permissible light intensity at which the CCDs become saturated. By lowering the EMCCD gain of the camera, the amount of light that can be detected without saturating the camera is increased, but the sensitivity for photons that can be detected for each fluorophore is decreased. When the camera gain is too low, the signal to noise ratio for single molecules is too low for fitting by a Gaussian function, thus rendering the fitting of individual spots with high accuracy impossible.

As a real example of the density of fluorophores that can be used and yet still find acceptable localizations of spots, we return to the super-resolution images produced by imaging the photobleaching of in vitro microtubules labeled with tetramethylrhodamine. If the number of fluorophores localized is plotted as a function of frame, we see that the number starts out low, increases to a peak, and then steadily decreases (see Figure 3.5). The starting, low number of spot localizations can be explained by assuming that the microtubules are overly labeled with fluorophores. As the fluorophores photobleach, the density of fluorophores decreases, and the ability to localize spots increases until a best fluorophore density is reached. The fluorophores then continue to photobleach so that the number of fluorophores localized per frame begins to decrease because the system is running out of available, active fluorophores. By knowing the average fluorescence output per frame of a single fluorophore, the total number of fluorophores per length of microtubule can be estimated. In Figure 3.5, the maximum fluorophores-localized-per-frame occurs at a fluorophore density of one fluorophore per 14 nm of length of microtubule.
We note that it is difficult or impossible (except in the case of blinking quantum dots) to localize as many apparent fluorophores as can be localized using other super-resolution techniques because fluorophores in those cases can be cycled on and off many times. We also note that background spots, such as fluorophores bound non-specifically to the coverslip surface, will tend to stand out more since individual fluorophores in crowded regions will not be identified as easily as those in less crowded regions.

3.5 Summary and conclusions

Notwithstanding some limitations, we believe our technique is useful because it requires no special laser cycling equipment and is compatible with all fluorophores that are sufficiently bright. Our width measurements on microtubules imaged using PhILM clearly show an improvement in resolution over using standard fluorescence microscopy. While the technique is limited in its ability to localize as many fluorophores as techniques involving photoactivation and suffers from additional shot noise (although these effects are reduced using techniques in Chapter 4, where a powerful combination of PhILM and other labeling techniques is introduced), it does have advantages, such as being compatible with many more fluorophores and not requiring special equipment. Thus, by applying the PhILM algorithm to data that can be taken on instrumentation that is already found in many labs, super-resolution microscopy is immediately available to a much broader range of users.

Additional methods

Spot detection algorithm. We used the following algorithm to automatically detect spots in the backwards-subtracted movie that correspond to photobleaching events:

1. Change all pixel values that are below zero to zero. This is done so that transition-to-bright events (which appear as dark spots in the image) do not interfere with finding spots that correspond to photobleaching events.
2. Dilate the image. To dilate the image, each pixel’s intensity value is replaced with the greatest intensity value of the eight nearest neighbors, or it is unchanged if the pixel intensity is greater than that of any one of the eight nearest neighbors. The
image might have to be dilated twice (i.e., apply this step twice) for very low signal to noise ratios.

3. Find the positions of all pixels that were not affected by the dilation. These are the local maxima.

4. Determine the average pixel intensity of all pixels within the radius $R$ (generally $R$ is chosen to be three pixels, which encloses the diffraction limited spot) of a given local maximum and call it the “spot average pixel intensity.”

5. Determine the average pixel intensity of all pixels between $R$ and $R + dR$ ($dR$ is generally chosen to be one pixel) around the local maximum and call it the “average local background intensity.”

6. Subtract the local background intensity from the spot average pixel intensity. Call it the “background-subtracted average spot intensity.”

7. Compare this with a chosen threshold value. Local maxima with values that are above the chosen threshold are considered “real” spots.

8. Perform an initial two-dimensional Gaussian fit on the spot. Reject spots that are either too narrow or too wide to be considered as reasonable spots corresponding to single molecules.

9. An output TIFF file is produced that can be compared with the original image file to check spot detection performance.

Spots corresponding to transition-to-bright-state events, which appear darker than background in the backwards-subtracted image, are detected in a very similar way:

1. Change all pixel values that are above zero to zero. This is done so that photobleaching events (which appear as bright spots in the image) do not interfere with finding spots that correspond to transition-to-bright-state events.

2. Dilate the image. To dilate the image, each pixel’s intensity value is replaced with the smallest intensity value of the eight nearest neighbors, or it is unchanged if the pixel intensity is less than that of any one of the eight nearest neighbors. The image might have to be dilated twice (i.e., apply this step twice) for very low signal to noise ratios.
3. Find the positions of all pixels that were not affected by the dilation. These are the local minima.

4. Determine the average pixel intensity of all pixels within the radius $R$ (generally $R$ is chosen to be three pixels, which encloses the diffraction limited spot) of a given local maximum and call it the “spot average pixel intensity.”

5. Determine the average pixel intensity of all pixels between $R$ and $R + dR$ ($dR$ is generally chosen to be one pixel) around the local maximum and call it the “average local background intensity.”

6. Subtract the local background intensity from the spot average pixel intensity. Call it the “background-subtracted average spot intensity.” Multiply by -1 to make the value positive.

7. Compare this with a chosen threshold value. Local minima with values that are above the chosen threshold are considered “real” spots.

8. Perform an initial two-dimensional Gaussian fit on the spot. Reject spots that are either too narrow or too wide to be considered as reasonable spots corresponding to single molecules.

9. An output TIFF file is produced that can be compared with the original image file to check dark spot detection performance.

*Polymerizing tubulin.* We prepared fresh polymerizing solution (1 mM GTP, 1 mM DTT, 50% glycerol in BRB80, pH 6.8). 2 μL biotinylated or fluorescent tubulin stock solution (20 μg/μL) was mixed with 5 μL native tubulin stock solution (10 mg/mL) on ice to prevent polymerization. 7 μL of prepared polymerizing solution was mixed with the tubulin solution. The resulting solution was incubated at 37°C for 15-30 minutes. 86 μL centrifugation solution (20 μM paclitaxel and 1 mM GTP in BRB80, pH 6.8) was then added to microtubules and gently mixed. The solution was centrifuged at 24°C at 15,000 g for 30 minutes. The supernatant was removed, and the pellet was resuspended in 100 μL centrifugation solution. The resulting microtubules were stored at room temperature.

*Preparing CLM K432-BIO-H6 kinesin.* CLM K432-BIO-H6 kinesin (a truncated form of human kinesin I) was designed to be composed of the first 432 amino acids of the popular
CLM K560 kinesin (22), a region that is biotinylated in *E. coli* (23, 24) and a six-histidine tag at the end for purifying on a nickel-NTA column (Qiagen). The following sequence was synthesized and inserted into a pET21a expression plasmid for us by GenScript, which contains an inserted, nearby Shine-Dalgarno site for improved expression (25):

5' -

```
GGATCCAAAAUAAGGAGGAAAAAATG GCCGATCTGGCGGAAAAGTAAATATC AAGAGTC
ATGTGCCGCTTTCTGCTTGCTGAATGAAGCGAGTGAAACCGCGCGCAAAAATTATATGCT
AAATTTCAGGGGAAGATACGGTCGTTGATTTGCCAACTTTACGCTGTTTGGACCGCTG
TTTCAGTCTTCGACCTTCAGGAACAGCTCTATAATGATGTGCTGCAAAACTGFTAAGG
GATTTGTCTGGAAGGGTATAAACGCCACTATTGTTGCTATGACCAACGCAAGCNAAG
CGATTTGTCTCAGCTCTCCGAGAAGTATATGGAATACACGACGAGGGGAGTTGAGTTCA
TCGTCGAAGACATTTTAAATTACATTACATTACCTAGCGATCTGACGCTTGACGTTTCGAA
CGAATCTGAGTGTGATGAAAGATGAAAGACCGCGTCTACGATGAAAGTCGAGCAGAA
CGTTTTGTCTGCTCTCCGAGAAGTATATGGAATACACGACGAGGGGAGTTGAGTTCA
TCGTCGAAGACATTTTAAATTACATTACATTACCTAGCGATCTGACGCTTGACGCTTG
AATGTCAGCTACTTGAATCTACCTCGGATAAAAATTCGCGATCTGCGAGCTTTCGAAA
CGAATCTGAGTGTGATGAAAGATGAAAGACCGCGTCTACGATGAAAGTCGAGCAGAA
CGTTTTGTCTGCTCTCCGAGAAGTATATGGAATACACGACGAGGGGAGTTGAGTTCA
TCGTCGAAGACATTTTAAATTACATTACATTACATTACCTAGCGATCTGACGCTTGACGCTG
AATGTCAGCTACTTGAATCTACCTCGGATAAAAATTCGCGATCTGCGAGCTTTCGAA
```

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AGATGATGAAACAAATTGAAGCAGATAAGTCAGGCGGTGAAAGCTATTCTGCGGGA
AGTGGTCAGCCGCTTGAATTTGATGAACCGCTGGTTGATCGAGCTGTCCCGAAACTT
GGTCACCACCATCATCATTAGCTT-3’

The plasmid was then used with the following protocol:

Lysis Buffer (other lysis, wash, and elution buffers can be used, but apparently it was best to avoid phosphate-buffered buffers to more accurately control ATP concentrations):

1. 100 mL water  
2. 6 g TRIS (50 mM)  
3. 1.5 g NaCl (250 mM)  
4. 20 mg MgCl2 (1 mM)  
5. 50 micromolar ADP  
6. 2 mM PSMF  
7. Adjust pH to 7.0

Wash Buffer:

1. 100 mL water  
2. 2.6 g TRIS (50 mM)  
3. 1.5 g NaCl (250 mM)  
4. 20.33 mg MgCl2 (1 mM)  
5. 50 micromolar ADP  
6. pH 8.0

Elution Buffer:

1. 100 mL water  
2. 6 grams (50 mM)  
3. 1.5 g NaCl (250 mM)  
4. 20 mg MgCl2 (1 mM)  
5. 3.4 g imidazole (500 mM)  
6. pH 7.2
Protocol

1. Transform BL21 (DE3) cells using manufacturer instructions. Plate cells, and incubate overnight. Using a single colony, inoculate 1 L of LB broth. Grow to $\text{OD}_{600} = 0.6-0.9$ at 37$^\circ$C and 250 rpm shaking. Induce protein expression by adding IPTG to 0.5 mg/mL. Incubate 4 h more at 37$^\circ$C with shaking. Harvest cells by centrifuging at 5000 g for 10 minutes. Store at -20$^\circ$C or 4$^\circ$C overnight.

2. Thaw kinesin pellets on ice. Add 100 mL of lysis buffer and mix by stirring and pipetting with a cell culture pipette tip.

3. Add 100 mg lysozyme. Incubate on ice for 30 minutes.

4. Sonicate with seven 10 s bursts with 10 s of cooling time between each burst. Sonicate at 200-300 W.

5. Centrifuge the solution at 30,000 g for 30 minutes to clear the lysate. Pour the supernatant into a new centrifuge tube.

6. Add 4 mL of Ni-NTA solution. Incubate for 60 minutes at 4$^\circ$C with gentle rocking.

7. Pour solution into a column. Let solution run through the column.

8. Wash with 50 mL of wash buffer (with 10 uM ATP added).

9. Add 30 mL of elution buffer (with 10 uM ATP added). Collect the first ten 0.5-1 mL fractions.

10. Quickly check which fractions contain protein using the Coomassie Plus™ assay (Thermo Scientific). Add 10 uL of kinesin solution to 250 uL of Coomassie Plus™. Pool the fractions that contain protein.

11. Add 50% sucrose (w/v) in BRB80 to make a final concentration of 10% sucrose.

12. Aliquot in 50 uL aliquots and freeze in liquid nitrogen. Store at -80$^\circ$C.

Imaging TMR-labeled microtubules in vitro. The starting labeling density was approximately 4:1 unlabeled:labeled tubulin monomers (Cytoskeleton, Cat. No. TL238 and TL590M). Microtubules were attached to a coverslip coated with a truncated form of kinesin. The microtubules were excited using 532 nm laser excitation and total internal reflection fluorescence (TIRF) microscopy. The microtubules were imaged in a solution containing beta-mercaptoethanol (Fluka) and oxygen scavenging reagents (PCA and PCD) to improve fluorophore stability (19, 20).
Imaging GFP-labeled microtubules in vitro.

1. Start with a 100% biotinylated PEG slide.
2. Flow in 5 mg/mL Neutravidin. Incubate 2 minutes.
3. Flow in 50 uL of (5 uL of biotin-microtubule solution + 44 uL of 10 mg/mL BSA in BRB80 + 1 uL of 2 mM paclitaxel). Incubate 5 minutes.
4. Flow through 100 uL of 10 mg/mL BSA in BRB80.
5. Flow in 100 uL of 100x dilution of stock CLM K560-GFP-H6 E215C kinesin (see [26]) in 10 mg/mL BSA in BRB80 and 20 uM paclitaxel. Incubate > 2 minutes.
6. Flow through 100 uL of 10 mg/mL BSA in BRB80.
7. Flow in 300 uL of (96 uL of 10 mg/mL BSA in BRB80 + 2 uL of 50 mg/mL PCA + 1 uL of 5 uM PCD + 1 uL of 2 mM paclitaxel).
8. Image with the stable Semprex stage, 488 nm TIRF excitation, Andor iXon+ camera.

Labeling axonemes. 4 Axonemes were purified as described elsewhere (22). 500 µl of stock axoneme solution (containing 50% glycerol) was added to 1 ml BRB80, pH 6.8, and kept on ice. The solution was then centrifuged at 15,000 g for 5 minutes at 4°C. The pellet was resuspended in 500 µl BRB80, pH 8.2, and kept on ice. 5 µl of 10 µM TAMRA (5-[and -6]-carboxytetramethylrhodamine, succinimidyl ester; Molecular Probes, Cat. no. C1171) dissolved in DMSO was added. The solution was incubated in the dark for 20 minutes at room temperature. The solution was then centrifuged at 15,000 g for 5 minutes at 4°C. The pellet was resuspended in 1.5 ml BRB80, pH 6.8, on ice. The centrifugation and resuspension was repeated two more times before finally centrifuging and resuspending in 100 µl of 1:1 BRB80:glycerol. The labeled axonemes were stored at -20°C.

4 Thanks to past and present lab members who went to the tedious work of purifying axonemes to produce general-use stocks for the lab.
Imaging rhodamine-labeled axonemes in vitro.

1. Start with a 100% biotinylated PEG slide (for protocol on preparing PEG slides, see [27]).
2. Flow in 5 mg/mL Neutravidin. Incubate 2 minutes.
3. Flow through 100 uL of 10 mg/mL BSA in BRB80.
4. Flow in 100 uL of 100x dilution of stock CLM K432-BIO-H6 kinesin in 10 mg/mL BSA in BRB80. Incubate > 2 minutes.
5. Flow through 100 uL of 10 mg/mL BSA in BRB80.
6. Flow in 50 uL of (5 uL of rhodamine-axoneme solution + 44 uL of 10 mg/mL BSA in BRB80). Incubate 5 minutes.
7. Flow in 100 uL of (95 uL of 10 mg/mL BSA in BRB80 + 2 uL of 50 mg/mL PCA + 1 uL of 5 uM PCD + 1 uL of stock BME [14 M]).
8. Image with a stable Semprex stage, 532 nm TIRF excitation, Andor iXon+ camera.

Imaging microtubules in cells using indirect antibody staining. COS-7 cells were seeded at low density in 35 mm glass bottom dishes and 2 mL DMEM with 10% FBS one day prior to antibody staining. The cell medium was replaced with OPTI-MEM (Gibco) 2 h prior to labeling. The medium was then removed and replaced with ~2 mL PBS. The PBS was replaced with 1 mL methanol that was stored at -20°C. The cells were fixed 5 minutes before replacing the methanol with 2 mL PBS with 0.1% Triton X-100. The cells were washed two more times with 2 mL PBS, waiting 5 minutes between washes. The PBS was then replaced with 2 mL blocking solution (3% [w/v] BSA and 0.5% Triton X-100 in PBS, filtered using 0.22 um syringe filter) and incubated at room temperature for 1 h. The blocking solution was then replaced with 2 mL of 1:100 dilution of DM1A anti-tubulin antibodies (Cell Signaling Technology, Cat. no. 3873S) in antibody dilution solution (0.4 g BSA and 120 μL Triton X-100 in PBS, filtered), and the cells were incubated at 4°C overnight. In the morning, cells were washed three times with 2 mL PBS, with 5 minutes between washes. The PBS was replaced with 1 mL 1:100 dilution of secondary antibody stock solution (either CF633 conjugated goat anti-mouse [2 mg/mL, Biotium, Cat. No. 20120-F], Alexa647 conjugated goat anti-mouse antibodies [1-2 mg/mL, Cell Signaling Technology, Cat. No. 4410], or rhodamine conjugated goat anti-mouse antibodies [1-2
mg/mL, Jackson Immunoresearch Laboratories, Cat. No. 115-026-062]) in antibody dilution solution and incubated 2 h at room temperature. The cells were again washed three times with 2 mL PBS, waiting 5 minutes between washes. Finally, the PBS was replaced with 100 mg/mL PCA and 20 μL of 5 uM PCD in 2 mL PBS immediately prior to imaging. CF633 was excited using 594 nm laser excitation in TIRF microscopy. Frames were acquired at 20 Hz, and because of the excellent brightness of the dye, we were able to use a very low EMCCD gain, which improved the dynamic range of the camera. Rhodamine was excited using 532 nm laser excitation, and Alexa Fluor 647 was excited with 633 nm excitation.
Figure 3.1. The principle behind PALM. A sparse subset of photo-activatable fluorescent molecules is activated (A and B) with a brief laser pulse at $\lambda_{\text{act}} = 405$ nm and then imaged at $\lambda_{\text{exc}} = 561$ nm until most are bleached (C). This process is repeated many times (C and D), with the sum of images producing (E and F). If the location of each molecule is determined by fitting the actual molecular image [(G), left] to a Gaussian, the molecule can be plotted [(G), right] as a Gaussian that has a standard deviation equal to the uncertainty in the fitted position. Repeating with all molecules across all frames (A’ through D’) and summing the results yields a super-resolution image (E’ and F’) in which resolution is dictated by the uncertainties as well as by the density of localized molecules. Originally published in (4) and used with permission.
The PhILM algorithm

Simple example:
1. Start with a two-fluorophore image sequence with photobleaching (frames 4 and 6):

2. Create the backwards-subtracted image sequence by subtracting frame 8 from 7 to get new frame 7’, 7 from 6 to get new 6’, etc.:

3. Locate spots in the backwards-subtracted image sequence. These show when and where photobleaching occurred (here, frames 4 & 6):

4. Localize the first photobleaching fluorophore by subtracting the average of frames 5-6 from the average of frames 1-4, then fitting to a weighted 2-dimensional Gaussian:

5. The center of the Gaussian fit is the location (within fit error) of the fluorophore. Repeat for the second fluorophore but subtract the average of frames 7-8 from the average of 5-6.

6. Plot the locations of the fluorophores in the new, super-resolution image!

Figure 3.2. Example of the PhILM algorithm applied to a simple, two-fluorophore movie. In a completely analogous way, fluorophores going from a dark state to a fluorescent state (as occurs with blinking fluorophores, photo-activation, or fluorophore binding events [see Chapter 4]) are also localized.
Figure 3.3. Creating the backwards-subtracted movie. To create the backwards-subtracted movie, frame 8 of the original movie is subtracted from frame 7 to produce 7’ of the backwards-subtracted movie, frame 7 is subtracted from frame 6 to produce 6’, etc. This simple example shows how photobleaching events and transition-to-bright-state events are represented by bright and dark spots, respectively, in the backwards-subtracted movie.
Figure 3.4. Application of PhILM to photobleaching microtubules. Microtubules were fluorescently labeled with tetramethylrhodamine and imaged under deoxygenating conditions. (Top) Sequence of frames demonstrating photobleaching over time. (Bottom left) Cropped section of frame #1. (Bottom right) Corresponding super-resolution image constructed using PhILM. The resulting super-resolution image was constructed from 3178 frames of data acquisition, 0.2 s per frame.
Figure 3.5. The optimal labeling density can be estimated as the fluorophore density when fluorophores localized per frame reaches a maximum. For the spots localized to produce super-resolution image in Figure 3.4, the maximum occurred near frame 700. The fluorophore density near frame 700 was estimated to be one per 14 nm length of microtubule. The heavy dark line indicates the frame intensity over time, which decays exponentially. A small drop in the intensity occurs near frame 800 due to a short interruption of data acquisition.
Figure 3.6. Distribution of spot localization errors. The spot localization error is calculated for two axes for each spot (e.g., x and y axes; 136,723 spots), and the errors for both axes are included in the histogram. Spots with calculated localization errors for either axis above 53 nm were not used to plot the super-resolution image (see Figure 3.4, bottom right).
Figure 3.7. Cross sectional widths of tetramethylrhodamine-labeled microtubules in vitro in normal and super-resolution PhILM images. An upper limit on the resolution (improvement) achieved can be estimated by fitting a straight line to short segments of the microtubules and plotting the distribution of fluorophores across the width of the microtubules. Here FWHM = 60 nm. The diameter of a microtubule is ~25 nm, but imperfect line fits, microtubule curvature, localization error, stage drift, etc., broaden the distribution. The FWHM in the regular image is 300 nm.
Figure 3.8. PhILM applied to GFP-labeled microtubules *in vitro*. Microtubules were labeled with GFP-kinesin. Although there was a clear improvement in the localization of the fluorophores, the number of localized spots was quite low, making it questionable whether we could apply PhILM to GFP, which is dim and photobleaches quickly.
Figure 3.9. Tetramethylrhodamine-labeled axonemes *in vitro*. (Left) Typical fluorescence image. (Right) PhILM image. Scale bar indicates 1 micron.

Figure 3.10. Segments of axonemes from Figure 3.9 were fit to straight lines, and the distribution of fluorescence perpendicular to the long axes of the axonemes was plotted to find the apparent width. 11 segments, each near 5 um in length, were fit this way to produce these plots.
Figure 3.11. Application of PhILM to photobleaching microtubules labeled with CF633 secondary antibodies in fixed COS-7 cells. (Left) Normal fluorescence image. Microtubule width at FWHM of the intensity = 500 nm. (Right) PhILM image, FWHM = 100 nm. Average spot localization error was 21 nm. Scale bar = 1 μm.
Figure 3.12. Additional example of PhILM applied to photobleaching microtubules labeled with CF633 secondary antibodies in fixed COS-7 cells. (Left) Normal fluorescence image. (Right) PhILM image. Scale bar = 1 μm.

Figure 3.13. Microtubule widths in images of microtubules labeled with CF633 conjugate secondary antibodies. Microtubule width at FWHM of the intensity = 500 nm. (Right) PhILM image, FWHM = 100 nm. Compare with Figures 3 and S5.
Figure 3.14. Microtubules in a fixed cell labeled with tetramethylrhodamine using secondary antibody staining. (Left) Normal fluorescence image. (Right) PhILM image created after substantially photobleaching the image. Scale bar = 1 μm.
Figure 3.15. Additional example of application of PhILM to lamB receptors labeled with blinking quantum dots on the surface of live *E. coli*. The cell outline, determined using bright field imaging, is shown in red. The top left image is the average of all frames of the movie (1000 frames, 3 ms per frame). Top right shows the standard super-resolution image produced using PhILM. Bottom image shows the “Z projection, maximum intensity” super-resolution image using spot localization via PhILM and is intended to highlight where receptors might be diffusing.
Figure 3.16. Application of PhILM to lamB receptors labeled with blinking quantum dots on the surface of live *E. coli*. (a) Sequence of images, frames selected at 499 frame intervals. (b) Average of all frames of the movie (1000 frames, 3 ms per frame). (c) Standard super-resolution image produced using PhILM. Average spot localization error = 18 nm. (d) Super-resolution image, but spots are plotted as “Z projections with maximum intensity,” i.e., only the maximum spot intensities are plotted—the super-imposed intensities do not add. Part (d) is intended to highlight where receptors might be diffusing, although spurious points can also become highlighted, due to the “maximum intensity” plotting method. Smaller spots indicate better localization accuracy. Scale bar = 1 μm.
Figure 3.17. Equation (3.5) estimates the localization error that can be achieved when there are several fluorophores crowded into a diffraction-limited area. The plot shows the predicted accuracy for $s = 125$ nm, $a = 100$ nm, and $N = 10,000$ photons. Increasing frame averaging, where $n$ is the number of frames averaged before and after a photobleaching event occurs, allows for smaller localization errors.
References for Chapter 3


Chapter 4: Super-resolution imaging using diffusive and transient fluorophore labeling

PhILM has many advantages such as the range of compatible fluorophores and simplicity of labeling samples. However, by using only photobleaching or blinking, it is limited by the number of nearby fluorophores that contribute to a noisy background, or worse, saturate the camera. In this chapter, a simple method for achieving PALM- and STORM-like data is introduced that is capable of signal-to-noise ratios similar to PALM and STORM, but it is much simpler to label the targets, requires no laser cycling equipment, and is applicable to a wider array of targets, including DNA. We combined two powerful approaches to image chromosomal DNA inside cells. In PAINT (1), the accumulated, stochastic binding events of fluorescent labels to an imaging target are localized. Surprisingly, PAINT has received little attention and so far has been limited to techniques for imaging in cell membranes (1, 2). In PhILM (photobleaching and intermittency localization microscopy), fluorophore transitions between dark and bright states (compatible with binding, photobleaching, photo-activation, blinking, etc.) are localized, even when fluorophore images overlap.

Combining the two techniques results in a robust microscopy that is faster than PAINT alone, requires less optimization, and corrects for cell autofluorescence. In addition, background noise due to fluorescent labels in solution can be virtually eliminated by using labels that fluoresce only when bound to the target. We refer to the technique as transient-labeling PhILM.

4.1 Description of the technique

By simply imaging targets, such as microtubules or other cell components, in a solution containing fluorescent molecules that bind to the target, a low density of bound, fluorescing molecules can be achieved. A sequence of images is acquired in which the single frame acquisition time is slow compared to the time required for a fluorescent molecule to diffuse

1 Most of the results of this chapter have been submitted to Nature Methods for publication (11).
through the length of an image pixel. Fluorophores in solution thus appear as a diffuse fluorescent background in the image. However, fluorescent molecules that are bound to the target do not diffuse quickly through the solvent but, rather, are localized to a single location and contribute a strong, localized fluorescent signal. The bound fluorophores show up as single spots if other fluorophores are not simultaneously immobilized within ~200 nm. Then, the problem of crowded, simultaneously fluorescing fluorophores is avoided. The resulting movie file is analyzed using the PhILM algorithm described in Chapter 3 to create a super-resolution image.

4.1.1 Using labels that do not unbind

Super-resolution images can be created if the fluorophores bind permanently, i.e., a fluorophore diffuses through the solvent until it binds, but does not dissociate (at least, not on time-scales much shorter than the experiment). In this case of purely diffusion-based labeling, the target is initially unlabeled. Fluorophores in solution begin to bind to the target over time. The bound fluorophores eventually photobleach, which helps to maintain a low density of bound, fluorescent fluorophores, as new fluorophores from solution continue binding to the target. If the binding events are separated enough in time and space, the fluorophores can be localized to create super-resolution images.

4.1.2 Using labels that bind transiently

In the case that the fluorophores bind transiently to the target, this technique provides some advantages over any previous super-resolution techniques. Photobleached fluorophores can dissociate from the binding site and be replaced by non-photobleached labeling fluorophores. In previous techniques, there was no way to replace permanently spent fluorophores. In essence, then, a single binding site can potentially be localized an unlimited number of times, as long as there are non-photobleached fluorophores available in the surrounding solution. The density of labeling fluorophores can also be controlled by limiting the number of fluorophores in solution or by adding competing, non-fluorescent labels. Lower densities of attached fluorophores result in higher fluorophore localization accuracy because there is less background fluorescence. However, they require longer times to localize the same number of fluorophores. Higher densities allow for faster
construction of a super-resolution image and require analysis using PhILM. Therefore, the labeling density can be modified, depending on the needs of the required application.

Another advantage of using transient labeling is that potentially every solvent-exposed binding site can be localized. This is not possible using previous techniques because of the finite size of bound labeling antibodies (which might not quickly unbind) or fluorescent proteins that might block nearby binding sites, pre-photobleaching of labeling fluorophores that bind and do not release, separation distance requirements based on fluorophore-fluorophore interactions like quenching, etc.

Another potential use of the technique that can be noted is the ability to calculate off rates. If the number of fluorophores bound is low and the photobleaching lifetime of the fluorophore is much longer than the bound state lifetime, the off-rate of the bound ligand can be measured by finding the distribution of times that ligands stay bound. This, however, is beyond the scope of this thesis.

4.1.3 Using labels that activate upon binding

The diffusion based labeling scheme is especially useful if the fluorophore does not fluoresce until it binds to the target. In this way, background fluorescence that would normally be contributed by diffusing fluorophores is eliminated. There are some dyes and approaches that can be used to achieve this. Some examples include using Förster resonance energy transfer (FRET) techniques and DNA dyes. For example, in FRET, fluorophores are used in pairs. One fluorophore in the pair is known as the “donor,” and the other is known as the “acceptor.” The absorption and emission properties are chosen such that the donor absorbance maximum is blue-shifted from that of the acceptor, and the donor’s emission maximum roughly matches the absorbance maximum of the acceptor. When the donor is excited by laser excitation and is in close proximity (i.e., within a few nanometers) of the acceptor, resonant energy transfer can occur, and the acceptor will emit light. If the two are not in close proximity, only the donor will emit light. If the acceptor is bound to the structure of interest and the donor only binds transiently (or vice versa), then emission of the acceptor can be used to produce super-resolution images with virtually no background due to the reporter dyes in solution. As a side benefit, if the donors are initially
bound to the target and the acceptors are diffusing in solution, the donor emission can be used to create a normal-resolution image that can be compared with the super-resolution image that is constructed from the acceptor emission. A drawback is that the donor will eventually photobleach.

In a slightly different approach, the “split GFP” fusion protein does not fluoresce until it binds to its missing, deleted peptide, which is added to the imaging solution (3). This is advantageous because the label is genetically encoded (and the missing peptide can be as well). However, it does suffer from weak fluorescence intensity, and the binding is apparently irreversible.

Many dyes that are used to directly label DNA, such as YOYO, SYTO, LOLO, etc., do not strongly fluoresce until they actually bind to DNA. Thus, many DNA dyes can be used in diffusive labeling for super-resolution imaging with virtually no background contribution due to fluorophores in solution. We used these to image DNA attached to glass and DNA in fixed cells, which is described below.

4.2 Some applications

4.2.1 Imaging microtubules using fluorescent paclitaxel

As a first experiment in diffusive, transient labeling, microtubules were imaged in nanomolar solutions of fluorescent paclitaxel. Paclitaxel, also known by the trade name “Taxol,” is an anti-cancer drug that binds to microtubules to inhibit cell division. Fluorescent paclitaxel is available commercially for research purposes. We used 7 nM Oregon Green 488 conjugated to paclitaxel (Invitrogen, Cat. no. P22310) to image microtubules bound to coverslips via a layer of kinesin. Besides paclitaxel, the imaging solution also contained bovine serum albumin (BSA), phosphate buffered saline (PBS), PCA, and PCD. PCA and PCD were used as an oxygen scavenging system (4) to decrease the photobleaching rate of the Oregon Green 488. As can be seen in Figures 4.2 and 4.3, the improvement in resolution was excellent. The average localization accuracy of a single dye was 10 nm, as determined using the PhILM program. The average fluorescence collected
from a single spot during a frame was 150 photons, which predicts a localization error of ≥11 nm and is close to the 10 nm error found using PhILM.

It was also noted that the off-rate appeared to be increased by increasing the laser excitation intensity. For a methanol-fixed COS-7 cells labeled with a solution of 350 nM Oregon Green 488 paclitaxel with 25 nM PCD and 2.4 mg/mL PCA in PBS, the intensity of the image slowly decreased under laser excitation, indicating that the bound paclitaxel was photobleaching. However, after a period of decreasing image intensity, the laser intensity was increased. To our surprise, the image intensity steadily increased (see Figure 4.1). We attributed this to photobleached paclitaxel unbinding from the microtubule and being replaced by non-photobleached paclitaxel from solution. After replacing the solution with PBS, the image intensity decreased quickly because there were no non-bleached fluorophores to bind to the microtubules.

Unfortunately, it was found during the in vitro microtubule imaging experiment that as the experiment continued for only a few minutes, the dyes rather suddenly became dim. Further imaging experiments suggested that the problem lay in using the PCA-PCD deoxygenation system. Although bright fluorescence was restored after flowing in fresh imaging solution, this severely affected our ability to image many fluorophores, which would be useful for building up a higher quality image. We also tried the same experiment using BODIPY 564/570 paclitaxel (Invitrogen). Unfortunately, in this case we found an excessive amount of non-specific binding of the dye to the cover slip surface.

4.2.2 Imaging microtubules using streptavidin S45A

We thus wished to find a system that was able to continue for much longer experiment times in order to localize more fluorophores. At the same time, we wished to find a more generalizable labeling scheme since the paclitaxel-microtubule system is limited to tubulin binding. To this end, we investigated using streptavidin as a means of transiently attaching fluorophores to structures. Streptavidin is a tetrameric protein with four binding sites that binds extremely tightly to its ligand, biotin. There are already many ways developed to label structures with biotin, including genetic methods. The strong binding is useful in many labeling reactions because the binding is considered to be virtually irreversible.
However, by mutating only a single amino acid (serine at residue #45 to alanine, denoted S45A), the dissociation constant is increased 1700 fold (5). At 37°C, the half-life of the bound state was measured to be 14 s (5). Because streptavidin has four binding sites, it can be used to simultaneously bind to a target structure that is conjugated with biotin as well as fluorophores that are conjugated with biotin. That is, it can be used as the “glue” for attaching fluorophores to target structures. By using the weakly binding streptavidin S45A mutant, it is then possible to transiently bind fluorophores to any structure of interest that has biotin on its surface.

We used the streptavidin S45A mutant to transiently label biotinylated microtubules attached to glass and create super-resolution images. Streptavidin S45A was prepared by expression in E. coli and purification as explained in the “Additional methods” section at the end of this chapter. Biotinylated microtubules were attached to a coverslip coated with truncated kinesin, a protein that binds to microtubules. 13 nM streptavidin S45A and 52 nM DNA oligomers (conjugated with biotin and Atto647N) were added to the chamber, and the microtubules were imaged. Fluorophores bound quite specifically to the microtubules, but a small fraction also bound non-specifically to the cover slip surface. The PhILM algorithm was applied to the resulting image files, and an example resulting super-resolution image is shown in Figure 4.6. The average fluorophore localization error was 16 nm.

4.2.3 Imaging chromosomal DNA

We chose to investigate super-resolution imaging using DNA dyes in solution. We began by imaging lambda DNA attached to a cover slip that was coated with poly-L-lysine. The DNA was flowed across the cover slip surface, so the DNA appeared as stretched out lines on the surface when imaged. We used nanomolar concentrations of SYTO 16 in 3 mg/mL BSA. Adding BSA was important for slowing the diffusion rate of the dyes (so that binding events were more distributed in time) and for blocking non-specific binding. Because the dyes bind quite tightly, we started imaging the DNA slightly after the dye was added so that

Thanks go to Eli Rothenberg for carrying out the DNA labeling experiments.
many of the binding sites were not filled at the beginning of data acquisition. We found a clear enhancement in resolution, and the average localization error of a spot was 36 nm. See Figure 4.7.

We also applied the technique to DNA in cells. HEK 293 and HeLa cells were seeded on glass-bottom petri dishes, fixed with methanol, and imaged in dilute solutions of DNA dye (see Figure 4.8). In this case, we again used SYTO 16 at nanomolar concentrations and 3 mg/mL BSA. The average localization error of single fluorophores in figure 6c was 24 nm. This represents a new method for super-resolution imaging of chromosomal DNA in a cell.

4.3 Some limitations
This technique is primarily limited by three sources: the on-rate of binding of fluorophores to the target, non-specific binding of fluorophores, and the target must be solvent accessible. If the fluorophores have a slow on-rate, then it can be some time before enough fluorophores have bound to create a decent super-resolution image. If imaging speed is paramount, then super-resolution imaging techniques such as dSTORM (6) and Blink Microscopy (7) that involve fluorophore blinking, or a combination of the techniques, are likely more appropriate. Diffusion based labeling can also present a problem if the fluorophores bind non-specifically to unintended targets, such as the coverslip surface in the case of BODIPY 564/570 Taxol, mentioned above. This is often not such an issue in other imaging techniques since excess fluorophores that are not strongly bound to the target can be washed away and no longer bind loosely with surrounding targets.

4.4 Conclusions
In this chapter it has been shown that by using weak solutions of fluorescent dyes in solution in combination with PhILM analysis, we can achieve excellent super-resolution imaging that surpasses that achievable using photobleaching and blinking alone. Also, by using fluorescent labels that bind only transiently with the target structure, the target can in principle be imaged indefinitely, localizing all of the binding sites infinitely many times. The technique lends itself well to a wide variety of fluorescent labels, making it, when combined with PhILM, a very robust and powerful approach to doing super-resolution microscopy. We have presented several new methods for transient labeling, including
methods for labeling microtubules, biotinylated structures, and chromosomal DNA in a cell. Given that only recently has DNA been imaged at super-resolution (using the blinking of YOYO dyes with DNA bound to glass; see [8, 9]), this last application might be particularly important. The labeling methods demonstrated are straightforward to perform and should make it possible for a much larger number of investigators to immediately apply super-resolution imaging to a broader range of targets and applications by using commercial dyes and equipment that are already found in many labs.

4.5 Additional methods

Imaging microtubules using fluorescent paclitaxel. Yellow-green fluorescent beads (Invitrogen, Cat. no. F-8787), used as fiduciary markers, were diluted in water and 12 mM HCl. The beads were flowed into a sample chamber and incubated >5 minutes. The chamber was then washed with 100 μL BRB80. 20 μL of 1 mg/mL kinesin was then added to the chamber and incubated for 5 minutes. The chamber was then washed with 100 μL BRB80, and 20 μL of microtubules diluted in BRB80 was added to the chamber and incubated for 5 minutes. Finally, 100 μL of imaging solution, consisting of 96 μL BRB80, 1 μL 700 nM Oregon Green Taxol (Invitrogen, Cat. no. P22310), 1 μL of 5 uM PCD, and 2 μL of 50 mg/mL PCA (pH 7.4), was flowed into the chamber. Imaging was done using 488 nm laser excitation. Unfortunately, it was found that as the experiment continued for only a few minutes, the dyes rather suddenly became dim. Although bright fluorescence was restored after flowing in fresh imaging solution, this affected our ability to image many fluorophores, which would be useful for building up an even higher quality image. We therefore tried the same experiment using BODIPY 564/570 paclitaxel (Invitrogen) to test whether or not this was a fluorophore-specific problem. Unfortunately, in this case we found an excessive amount of non-specific binding of the dye to the coverslip surface.

Preparation of streptavidin S45A. The streptavidin S45A plasmid was a gift from Patrick Stayton’s lab and has been described previously (5). Streptavidin S45A was prepared by expression in E. coli and purification in a modification of the method presented by Howarth et al. (10), which follows:
*Eschericia coli* BL21 (DE3) cells were transformed with the plasmid and grown on agar plates containing ampicillin overnight. A 15 mL overnight culture of LB with 50 μg/mL ampicillin was inoculated and grown at 37°C with shaking.

1.5 L of LB medium and 50 μg/mL ampicillin was inoculated using the overnight culture. The culture was grown with shaking at 37°C for 4-5 hours until OD600 = 0.7. Then protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to make a final concentration of 100 μg/mL. The cells were grown for another 4 h, at which point the cells were harvest by centrifuging at 8000 g for 5 minutes. The supernatant was discarded, and the cell pellet was stored overnight at 4°C.

The cell pellets were treated using B-PER (Thermo Scientific, Cat. no. 90078), following the manufacturer’s instructions. The cell pellet was resuspended in 10 mL of B-PER. 10 mL more B-PER were added, and the solution was left to incubate 10 minutes on the bench top with occasional swirling. 100 mL of inclusion body wash buffer (50 mM TrisHCl, 0.5% Triton-X100, 100 mM NaCl, 1 mM EDTA, 0.1% [w/v] sodium azide, and 1 mM DTT, pH 8.0) was then added, and the solution was mixed well. The solution was then centrifuged at 27000 g for 10 minutes. The supernatant was discarded. The pellet was then resuspended in 10 mL of inclusion body wash buffer, and 90 mL of additional inclusion body wash buffer were added. The solution was centrifuged at 15000 g for 10 minutes. The supernatant was discarded, and the pellet was again resuspended in 10 mL plus 90 mL of inclusion body wash buffer and centrifuged. After this third wash, the pellet was resuspended in 10 mL plus 90 mL of inclusion body wash buffer that was missing Triton-X100. The solution was centrifuged again at 15000 g for 10 minutes. The supernatant was discarded. The pellet was then dissolved in 5.5 M guanidine hydrochloride in water at pH ~1.5. 250 mL of PBS was cooled to 4°C. Using a magnetic stir bar, the PBS was stirred rapidly so that the vortex reached the top of the stir bar. The guanidine solution was then slowly added dropwise to the fastest moving part of the solution. The stir rate was reduced, and the solution was stirred overnight at 4°C.

In the morning, the solution was centrifuged at 17700 g for 15 minutes at 4°C. The supernatant was then returned to stir at 4°C while the pellet, which was assumed to contain
misfolded and aggregated protein, was discarded. 62.8 g of ammonium sulfate was then added, approximately 10 g at a time, to the stirring solution. The solution was stirred at 4°C for 3 h. The solution was then passed through filter paper (Whatman 42) using vacuum filtration. The flow-through was returned to stirring at 4°C. 59 g of additional ammonium sulfate was added all at once to the solution. The solution continued to stir for three hours. The solution was then centrifuged at 17700 g for 15 minutes at 4°C. The supernatant was discarded, and the bottle was allowed to drain upside down on paper towels for approximately 2 minutes. The pellet was then resuspended in 4 mL of PBS. The solution was centrifuged at 14000 g for 5 minutes at 4°C. The supernatant was kept while the pellet was discarded. The OD280 was then measured by using PBS as a blank and assuming 0.1 mg/mL streptavidin for OD280 = 0.355. The total yield measured in this way was 35 mg.

The solution was then further diluted in PBS to a final concentration of 0.7 mg/mL and stored at 4°C.

_Gel electrophoresis of streptavidin S45A product_. See Figure 4.4. The following lanes were prepared for running a 15% poly-acrylamide gel:

Lane 1, pre-induction bacteria proteins. 50 uL of bacteria was taken from the culture. The bacteria were spun down, and the supernatant was removed. It was replaced with 16 uL of PBS and 4 uL of 5x SDS-PAGE gel loading buffer. The sample was boiled at 95°C on a heat block for 5 minutes. The tube was spun briefly to removed condensation from the lid and ensure that the solution was well mixed. 15 uL of the solution was then added to one lane of the gel.

Lane 2, post-induction bacteria proteins. 50 uL of bacteria was taken from the culture after inducing for four hours with IPTG. The bacteria were spun down, and the supernatant was removed. It was replaced with 16 uL of PBS and 4 uL of 5x SDS-PAGE gel loading buffer. The sample was boiled at 95°C on a heat block for 5 minutes. The tube was spun briefly to removed condensation from the lid and ensure that the solution was well mixed. 15 uL of the solution was then added to one lane of the gel.
Lane 3, purified streptavidin S45A protein. The final concentration of the protein was estimated to be 0.7 mg/mL in PBS by measuring the absorbance at 280 nm. 16 uL of the protein solution and 4 uL of 5x SDS-PAGE gel loading buffer were mixed. The sample was boiled at 95°C on a heat block for 5 minutes. The tube was spun briefly to removed condensation from the lid and ensure that the solution was well mixed. 15 uL of the solution was then added to one lane of the gel.

Lane 4, commercial strepavidin (Thermo Scientific, Cat. no. 21122). 2 uL of 5 mg/mL streptavidin was mixed with 14 uL of PBS. 16 uL of the protein solution and 4 uL of 5x SDS-PAGE gel loading buffer were mixed. The sample was boiled at 95°C on a heat block for 5 minutes. The tube was spun briefly to removed condensation from the lid and ensure that the solution was well mixed. 15 uL of the solution was then added to one lane of the gel.

Lane 5, commercial NeutrAvidin (Invitrogen, Cat. no. A2666). 2 uL of 5 mg/mL streptavidin was mixed with 14 uL of PBS. 16 uL of the protein solution and 4 uL of 5x SDS-PAGE gel loading buffer were mixed. The sample was boiled at 95°C on a heat block for 5 minutes. The tube was spun briefly to removed condensation from the lid and ensure that the solution was well mixed. 15 uL of the solution was then added to one lane of the gel.

4 uL of gel loading buffer was added to each of the remaining, empty lanes.

Testing for binding of streptavidin S45A to biotin. I checked to see whether or not it would bind to a surface coated with biotinylated bovine serum albumin (BSA) and a DNA construct with a biotin tag at one end and an Atto647N dye at the other. I added 10 uL of 5 mg/mL BSA-biotin to two flow chambers. I waited > 5 minutes, and then I washed with 100 uL of phosphate buffered saline (PBS) solution. To one chamber, I added 26 nM of Atto647N-DNA-biotin with 3% BSA in PBS. To the other chamber, I added 26 nM Atto647N-DNA-biotin with 53 nM streptavidin S45A and 3% BSA in PBS. I imaged both slides on the MegaMan 1 setup using the HeNe laser. Results showed that addition of streptavidin S45A caused many fluorophores to bind to the surface (see Figure 4.5).
**Polymerizing tubulin.** We prepared fresh polymerizing solution (1 mM GTP, 1 mM DTT, 50% glycerol in BRB80, pH 6.8). 2 μL biotinylated or fluorescent tubulin stock solution (20 μg/μL) was mixed with 5 μL native tubulin stock solution (10 mg/mL) on ice to prevent polymerization. 7 μL of prepared polymerizing solution was mixed with the tubulin solution. The resulting solution was incubated at 37˚C for 15-30 minutes. 86 μL centrifugation solution (20 uM paclitaxel and 1 mM GTP in BRB80, pH 6.8) was then added to microtubules and gently mixed. The solution was centrifuged at 24˚C at 15,000 g for 30 minutes. The supernatant was removed, and the pellet was resuspended in 100 μL centrifugation solution. The resulting microtubules were stored at room temperature.

**Imaging microtubules using streptavidin S45A.** 10 μL of 1 mg/mL truncated kinesin solution was added to a flow chamber and incubated 5 minutes. 50 μL of blocking solution (50 μL 20 mg/mL casein, 1 μL 10 mM ADP) was flowed into the chamber and incubated 5 minutes. The chamber was then washed with 100 μL of 100 uM ADP in PBS, pH 8.2. 100 μL of microtubule solution (95 μL 100 uM ADP in PBS, pH 8.2; 1 μL of 20 uM paclitaxel [Cytoskeleton] in DMSO; 5 μL of solution of biotinylated microtubules) was added, and the chamber was incubated 5 minutes. 100 μL of imaging solution (100 μL 100 uM ADP in PBS, pH 8.2; 0.5 μL 5 uM [biotin]-DNA-[Atto647N]; 4 μL of 1.3 uM streptavidin S45A in 10% sucrose in PBS, pH 8.2; 1 μL of 20 uM paclitaxel [Cytoskeleton] in DMSO) was then added. Atto647N was imaged using 633 nm laser excitation in TIRF microscopy with a 0.5 s frame acquisition time.
Figure 4.1. Increasing laser power increased paclitaxel binding to microtubules in cells in 325 nm Oregon Green 488 paclitaxel. (Top) For moderate laser power, the cell photobleached over time, with a decreasing mean pixel intensity. (Bottom) Doubling the laser power increased the mean pixel intensity over time. This was possibly due to laser induced unbiding of photobleached paclitaxel from the microtubules and subsequent binding of unbleached fluorophores.
Figure 4.2. Imaging results for Oregon Green 488 paclitaxel on microtubules. Individual spots were localized with an average 10 nm localization error.

Figure 4.3. Microtubule widths in images of microtubules labeled with Oregon Green 488 Taxol (Invitrogen).
Figure 4.4. Streptavidin S45A purification gel. Lanes 1 and 2 show the pre- and post-induction bands of cell lysates. Lane 3 shows our final streptavidin S45A product. Lane 4 shows commercial streptavidin (Thermo Scientific, Cat. no. 21122). Lane 5 shows commercial NeutrAvidin (Invitrogen, Cat. no. A2666), which apparently is less pure than the streptavidins. It is immediately apparent that the IPTG induction worked very well. The lane for the purified protein has only one band, and it apparently has the same size protein as the lane to its right, which is commercial streptavidin. Incidentally, the NeutrAvidin lane has four distinct protein bands.
Figure 4.5. Verification that streptavidin S45A binds biotin. (Left) Result of adding dye, but no streptavidin S45A. It shows that there are only a few, non-specifically attached dyes on the surface. (Right) Result of adding dye and streptavidin S45A.
Figure 4.6. Transient labeling using streptavidin S45A. (a) Image sequence with frames selected at intervals of 499 frames. (b) Average image of all frames in the image stack. Very bright spots are fluorescent 40 nm dark-red beads (Invitrogen, Cat. no. F-8789), which were used as fiduciary markers. (c) Resulting super-resolution image produced using PhILM analysis. Average spot localization error = 16 nm. Frame acquisition time was 0.5 s. 2000 frames were analyzed. Scale bar = 1 μm.
Figure 4.7. Lamda DNA on glass and labeled with SYTO in solution. (Left) Average of all frames of movie. (Right) Result of PhILM analysis. In these images, drift correction was not used. Scale bar = 1 μm.
Figure 4.8. Super-resolution imaging of DNA in a HEK cell. DNA was labeled using a dilute solution of SYTO dye. (a) Image sequence with frames selected at 199-frame intervals. (b) Average of all fluorescence imaging frames (green) merged with a brightfield image of the cell showing the outline of the cell (blue). (c) PhILM image. Average spot localization error was 24 nm. (d) Zoomed-in portion of part (c). The PhILM image was constructed from 1000 image frames, 0.2 s per frame. Scale bar = 1 μm.
References for Chapter 4


Curriculum Vitae
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Research aims
Improve and apply single-molecule techniques in basic science with an emphasis in medicine. Develop further techniques for studying chromosomal DNA in cells with super-resolution fluorescence imaging. Single-molecule characterization of membrane-bound receptors.

Education
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Publications


Patents
- Provisional patent application #61307440, “Photobleaching and Intermittency Localization Microscopy.”

Awards
- Best basic science poster, University of Illinois College of Medicine Research Symposium (2010).
- Utah State University Physics Department Undergraduate Departmental Scholarship (January 2001).
• University of Utah Merit Scholarship with Presidential Honors (May 1997, offered but not accepted).
• Brigham Young University Heritage Scholarship (May 1997, offered but not accepted).
• National Merit Finalist (May 1997).

Abstracts


Paul D. Simonson, Steven S. Rosenfeld, Paul R. Selvin, “Decoding Kinesin’s Asymmetric Walk Through Neck Rotation Experiments,” University of Illinois College of Medicine Spring Research Symposium, Urbana, IL, April, 2008.


Paul D. Simonson, Enrico D'Amico, and Enrico Gratton, “Modulation of an optical needle’s reflectivity alters the average photon path through scattering media,” UIUC College of Medicine Research Symposium, Hawethorne Suites, Champaign, IL, April 22, 2005.


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