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Triplet-state mediated super-resolution imaging of fluorescently-labeled ligands on gold nanorods: a single molecule, single particle approach

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Triplet-state mediated super-resolution imaging of fluorescently-labeled ligands on gold nanorods: a single molecule, single particle approach

by

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Dedication

To my family, thank you for all the love and support throughout the years.

To my brother, Dr. Derek Blythe, thank you for paving a great path of educational and professional accomplishments that I wanted to follow (although you never gave me a choice in the matter).

To my adviser, Dr. Kallie Willets, thank you for the guidance and encouragement over the years that lead to the great experiments and results discussed in this dissertation.

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To Dr. Eric Titus, thank you for sharing this adventure, in both life and research, with me.
Triplet-state mediated super-resolution imaging of fluorescently-labeled ligands on gold nanorods: a single molecule, single particle approach

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Super-resolution imaging was used to study the binding interactions between fluorescently-labeled double-stranded DNA (dsDNA) ligands and gold nanorods (AuNRs). The dsDNA is attached to AuNRs via a thiol linker and the fluorescent label served as a reporter molecule. A triplet-state mediated technique was used to modulate emission from the reporter molecules such that ideally only one was emitting at a time. The steady AuNR luminescence contribution was subtracted from the fluorophore emission, and the result was fit to a model function to extract the centroid position of the reporter molecule, which maps the apparent location of the dsDNA with respect to the AuNR surface. With this technique, we have observed instances where the majority of ligands bind preferentially at the ends of the AuNR, one end of the AuNR has more binding than the other, or the binding is spread evenly over the surface of the AuNR. We hypothesize that the differences in fluorophore localization between functionalized AuNRs was due to apparent binding heterogeneity, providing insight that is not attainable with bulk studies.

By mapping the apparent locations of the dsDNA bound to the AuNRs, we were also able to reconstruct the shape and orientation of the underlying AuNR, but the
size of the reconstructed image was almost always smaller than expected, when compared to dimensions obtained using electron or atomic force microscopy. To address this mismatch we tested several hypotheses. We used a better model (a dipolar emission model instead of two-dimensional Gaussian) to fit the AuNR luminescence contribution and the improved model led to more robust reconstructed images by allowing more centroid positions to pass the quality-of-fit criterion. We also altered the concentration of the fluorescently-labeled DNA available for binding and tested reporter molecules with different non-fluorescent lifetimes. These strategies aimed to lower the probability of reporter molecules emitting simultaneously, but these experiments did not fix the size mismatch. Nevertheless, under those different experimental conditions, we continually observed apparent binding heterogeneity of dsDNA across the surface of AuNRs based on the super-localization of the reporter molecules, indicating the power of this technique for observing nanoscale heterogeneity.
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Chapter 1: Introduction

1.1 Gold Nanorods and Their Applications

Gold nanorods (AuNRs) have become a widely used substrate in many research fields, such as bioimaging,\textsuperscript{1–5} theranostics,\textsuperscript{3,6–10} catalysis\textsuperscript{11,12}, and spectroscopy\textsuperscript{13–16}. For example, El-Sayed and coworkers have used AuNRs as a cancer therapy agent by functionalizing the particles with a targeting ligand to bind specifically to malignant cancer cells. Once targeted, the cancer cells were destroyed by utilizing the local heating properties of the AuNR using a technique called photothermal therapy.\textsuperscript{3} Several research groups have also shown some promising results towards using AuNRs as laser-triggered \textit{in vivo} drug delivery agents, where the drug of interest is usually tethered to the particle by a ligand.\textsuperscript{7,10,17,18} Due to the high surface area to volume ratio of AuNRs, they are well-suited to serve as multifunctional particles functionalized with both targeting ligands, for site specific binding to targets of interest, and drug ligands, for treatment, attached on the same surface.\textsuperscript{19,20}

The appeal of using these particles is based on both the relative chemical inertness of gold and the light-mediated properties that are exhibited by AuNRs. On the nanoscale, the AuNRs exhibit easily tunable localized surface plasmon resonances,\textsuperscript{6,8,21–23} photostability,\textsuperscript{24,25} and local heating properties.\textsuperscript{8,22} In order to increase the versatility of AuNRs in different research fields, they are often functionalized with different ligands that allow for them to be used in a variety of applications. Several examples are mentioned above, and in addition to those, the ligands can also be useful in reducing the \textit{in vivo} toxicity of the gold nanoparticle,\textsuperscript{1,5,6} and improving nanoparticle stability.\textsuperscript{1,2} The ability of AuNRs to be so widely used is based on the specific functionality of the ligands that are bound to the particles in these experiments.

1.2 Characterizing Functionalized Gold Nanoparticles

There are several bulk characterization techniques that can be used to study ligand binding on the surface of nanoparticles. These techniques are considered bulk because they are usually carried out on many nanoparticles in solution and are susceptible to averaging over any
heterogeneity that can occur on the nanoparticle surfaces. For example, dynamic light scattering (DLS) is used to track the changes in nanoparticle motion in solution due to ligand binding. The data processing involved with DLS also provides the hydrodynamic radius of the particle before and after ligand binding to show the increase in particle size due to the presence of the ligand.\(^{26-28}\) Another technique that is used is bulk fluorescence if the ligand is fluorescently labeled.\(^{26,29}\) However, this technique often involves removing the gold substrate before the fluorescence measurements are taken. In those cases, the fluorescently-labeled ligand is isolated from the gold particle by dissolving the gold or by breaking the bond attaching them together, which prevents the particles from quenching the fluorescence emission. The fluorescence from the remaining fluorescently-labeled ligands in solution is measured and the signal detected can be correlated to the concentration of ligands present. Zeta-potential measurements are also used to measure the change in particle surface potential before and after ligand binding.\(^{26,28,30}\) A change in the zeta-potential indicates a change in the nanoparticle surface chemistry caused by the ligands. Although these techniques provide proof that ligand binding has occurred, they do not provide insight into where the ligands are binding on the nanoparticle surface and whether any particle-to-particle binding heterogeneity exists.

**Characterizing Binding Heterogeneity**

The process of functionalizing AuNRs usually involves mixing the ligands and AuNRs in a solution and allowing the ligand to randomly bind *via* electrostatic or covalent interactions to the AuNR surface. When the ligands are randomly binding to the AuNR surface in solution it is non-trivial to dictate the binding location or the amount of binding that can occur on individual particles. Furthermore, chemically produced AuNRs are prepared with an initial stabilizing ligand to keep the particles soluble in water. Therefore, a ligand exchange is needed in order to coat the AuNR with another functional ligand.\(^{27}\) The level of difficulty in exchanging the initial stabilizing ligands varies depending on the AuNR surface and if the initial ligand binds more strongly to specific regions of the AuNR surface.\(^{27,31,32}\)
In some cases, preferential ligand binding will happen on the ends of the AuNRs and this phenomenon can be exploited to create chains of end-to-end linked AuNRs, verified using scanning electron microscopy, as depicted in Figure 1.1A.\textsuperscript{16,31,32} Other examples of possible heterogeneous binding on AuNRs are depicted in Figure 1.1, such as asymmetric binding (Figure 1.1B), non-uniform self-assembly (Figure 1.1C), or low binding (Figure 1.1D). Binding heterogeneity on the surface of nanoparticles has also been observed when using ligands labeled with two different fluorescent reporter molecules.\textsuperscript{29} In this case, the stoichiometry between the different ligands that were added in the solution was not preserved on the nanoparticle surfaces. Understanding the binding heterogeneity that can occur when different ligands are expected to bind to a AuNR in an equal ratio is important, especially when designing experiments that involve multifunctional nanoparticles. In order to fully understand how different ligands in solution bind to AuNRs on the nanoscale, we developed a super-resolution imaging approach that is sensitive enough to observe binding heterogeneity on individual AuNRs at the single molecule, single particle level.

Figure 1.1  Examples of ligand binding heterogeneity that can occur on AuNRs.

1.3 Super-Resolution Imaging

Defeating the Diffraction Limit of Light

For our experiments, we used fluorescently-labeled double-stranded DNA (dsDNA) as the ligand that binds to the AuNRs. Unfortunately, traditional optical techniques such as dark field microscopy (Figure 1.2A) and fluorescence microscopy (Figure 1.2B) do not provide the required resolution needed to map the location of the fluorescently-labeled dsDNA on the AuNR
surface. This is due to the diffraction limit of light and the Rayleigh criterion which limits optical resolution to approximately half the wavelength of light used for imaging. Techniques such as atomic force microscopy and scanning election microscopy (Figures 1.2, C and D) can provide images of the underlying AuNR but cannot provide the contrast needed to see the individual dsDNA.

![Figure 1.2 Images of different functionalized AuNRs taken with (A) dark field microscopy, (B) fluorescence microscopy, (C) atomic force microscopy, and (D) electron microscopy.](image)

In order to map the location of ligands bound to a nanoparticle using optical approaches, a super-resolution technique is necessary for overcoming the diffraction limit of light. There are several super-resolution techniques, such as stimulated emission depletion (STED) and structured illumination microscopy, that involve a more complicated microscopy setup to achieve sub-diffraction limited imaging.\(^{33-35}\) Another approach involves post imaging data processing that fits the point spread function (PSF) from a nano-emitter to a model function to extract the centroid position of the emitter with a precision of 10 nm or better.\(^{36-40}\) A two-dimensional Gaussian model function, Equation 1, is commonly used to fit the PSF, however more rigorous modeling functions are often necessary.\(^{41-47}\)

\[
I(x,y) = z_0 + I_0 e^{-\left[-\frac{1}{2}\left(\frac{x-x_0}{s_x}\right)^2 + \left(\frac{y-y_0}{s_y}\right)^2\right]} \tag{1}
\]
When using the two-dimensional Gaussian, \( I(x,y) \) is the spatially-dependent intensity, \( z_0 \) is the background intensity, \( I_0 \) is the peak intensity, \( s_x \) and \( s_y \) are the widths of the Gaussian in \( x \) and \( y \), and \( x_0 \) and \( y_0 \) is the centroid position. Figure 1.3 shows a depiction of the projected PSF of a nano-emitter before and after the two-dimensional Gaussian fit is applied and the extracted centroid position gives the approximate location of the origin of the emission.

![Figure 1.3 Depiction of the PSF fitting process when the model function used to calculate the centroid position is a two-dimensional Gaussian. From left to right: raw diffraction-limited emission projected into the \( x-y \) imaging plane, the same data projected in three dimensions with the \( z \)-dimension representing the emission intensity, and the 2-dimensional Gaussian fit to the emission data.]

**Ground State Depletion Microscopy**

In order to use the PSF fitting technique, the fluorescent labels on the dsDNA need to be modulated between fluorescent and non-emissive states such that, ideally, only one fluorophore is emitting at a time. Otherwise, upon excitation, all the fluorophores in the diffraction-limited spot would emit and the calculated centroid position would represent the intensity-weighted super-position of all the emission, as seen in Step A of Figure 1.4. In order to induce this photoswitching, we used a technique called Ground State Depletion Microscopy Followed By Individual Molecule Return (GSDIM).\(^{38,48}\) This technique relies on a fluorophore’s natural ability to intersystem cross into a non-emissive dark state from which it can eventually recover (Figure 1.5). Upon laser excitation, a fluorophore can be excited and then emit photons for fluorescence emission or a fluorophore can intersystem cross into the triplet-state. By supplying enough energy, *via* laser excitation, most fluorophores are forced into the triplet-state (once there other longer lived dark states may be accessed as well). We refer to this as the ‘off’ state because
the fluorophores are non-emissive, as depicted in Step B of Figure 1.4. Eventually, a single fluorophore will return from the non-emissive state to the ground state and become fluorescent, or in the ‘on’ state, as in Step C of Figure 1.4. The emission from the fluorophore in the ‘on’ state is then fit to Equation 1, as shown in Step D of Figure 1.4, to extract the location of the emitter with respect to the nanorod surface. The location of the fluorophore also indirectly provides the location of where the dsDNA was bound to the AuNR. This process is then repeated multiple times in order to probe the location of each fluorophore on the AuNR surface.

![Diagram](image)

**Figure 1.4** Depiction of fluorophores undergoing GSDIM while attached to an AuNR. In Step A the functionalized AuNR is excited *via* laser excitation, at this step it is likely that many molecules may fluorescence. After a short time period, Step B, the majority of the fluorophores are in the ‘off’ state due to intersystem crossing. In Step C ideally one fluorophore returns to the ‘on’ state to undergo the cycle of being excited and emitting photons. In Step D the PSF of the emitted photons are fit and the fluorophore’s centroid position is extracted. Steps B-D are repeated until each individual fluorophore has been localized.

When multiple fluorophores are located within a diffraction-limited spot, it is crucial to use a high enough laser power to force the majority of the fluorophores into the ‘off’ state, which
has a lifetime lasting milliseconds to seconds. Due to this fact, the majority of the fluorophores remain in the ‘off’ state and ideally one fluorophore at a time can relax back to the ground state and undergo multiple fluorescence cycles to produce many photons. Figure 1.5 shows a Jablonski diagram depicting the GSDIM process. After emitting many photons, the molecule can intersystem cross again or photobleach. This super-resolution technique is advantageous because only one excitation source is needed, for both fluorophore excitation and forcing intersystem crossing, and the fluorophores needed for this technique are relatively inexpensive. The experiments are done in a nitrogen environment to eliminate triplet-state quenching oxygen and promote a longer lived triplet state. Also, these experiments are done by using the highest intensity of laser power necessary to shelf the majority of the reporter molecules into the “off” state without causing structural changes to the underlying AuNR.

![Jablonski diagram of a fluorophore undergoing GSDIM. The fluorophore can be in the fluorescent ‘on’ state or in the non-emissive ‘off’ state.](image)

**Figure 1.5** Jablonski diagram of a fluorophore undergoing GSDIM. The fluorophore can be in the fluorescent ‘on’ state or in the non-emissive ‘off’ state.

### 1.4 Scope of This Work

The aim of this research is to use super-resolution imaging to study the binding interactions between AuNRs and fluorescently-labeled dsDNA. By using the centroid position of
the fluorophore emission we can map the apparent location of where a dsDNA bound to the AuNR surface. This super-resolution optical technique has allowed for the observation of the binding heterogeneity that occurs between AuNRs and ligands assembled in solution. Chapter 3 discusses the results of using this triplet-state mediated technique to map the location of dsDNA on the surface of AuNRs. We were able to observe binding heterogeneity as well as reconstruct the shape and orientation of the underlying AuNR. Correlated atomic force microscopy was used to verify the size, shape, and orientation of the AuNR. Unfortunately, the size of the reconstructed AuNR image was smaller than expected. Chapters 4 and 5 discuss several hypotheses that were tested to address the size mismatch issue. Chapter 4 focuses on the effects of using a better model to fit the AuNR luminescence on the resulting reconstructed image. If the AuNR contribution is poorly modeled then it can skew the fluorophore centroid positions towards the AuNR luminescence position, therefore shrinking the size of the reconstructed image. Chapter 5 discusses the results from altering the concentration of fluorescently-labeled dsDNA available in solution to bind to AuNRs. The chapter also focuses on changing the fluorophore used to label the dsDNA in order to achieve longer ‘off’ state lifetimes. Ideally, both of these changes should result in lowering the probability of having fluorophores emitting simultaneously and impacting the calculated super-position centroids. Lastly, Chapter 5 discusses the effects of using longer dsDNA, to increase the distance between the AuNR and the fluorophore, on the reconstructed images of labeled-AuNRs. Chapter 6 describes the effects that different objectives can have on the PSF of diffraction-limited dipolar-like emitters, such as AuNRs. In this Chapter we evaluate the images collected from several objectives and qualitatively decide which are best for collecting data involved in super-localization experiments. Finally, in Chapter 7 we provide a brief outlook for the future direction and experiments for this project.
1.5 References


Chapter 2: Methods

2.1 Gold Nanorods

Gold Nanorod Synthesis

Cetyltrimethylammonium bromide (CTAB) coated gold nanorods (AuNRs) used in all the experiments discussed in this dissertation were prepared using a seed mediated procedure.\textsuperscript{1–3} The chemicals used in this procedure were purchased from Sigma-Aldrich and 18.2 MΩcm nanopure water was used to make all the solutions. The seed solution was prepared by mixing 9.75 mL of 0.1 M CTAB, 0.25 mL of 0.01 M chloroauric acid (HAuCl\textsubscript{4}), and 0.6 mL of 0.01 M ice cold sodium borohydride (NaBH\textsubscript{4}) into a scintillation vial. The solution was mixed by inversion for two minutes, the resulting color was a light brown with a pinkish tint. The seed solution sat at room temperature for two hours to allow the seed formation to come to completion. Next, a AuNR growth solution was prepared by mixing 40 mL of 0.1 M CTAB, 2 mL of 0.01 M HAuCl\textsubscript{4}, 0.6 mL of 0.01 M silver nitrate (AgNO\textsubscript{3}), 0.8 mL of 1 M hydrochloric acid (HCl) and 0.32 mL of 0.1 M ascorbic acid in an Erlenmeyer flask. The solution was swirled briefly after the addition of each ingredient. Finally, 100 µL of a 10% seed solution (diluted with nanopure water) was added to the growth solution. The solution was swirled and allowed to sit at room temperature overnight to ensure the completion of AuNR formation. Extinction spectra, taken with a UV-VIS spectrophotometer, was used for bulk characterization of the AuNR solution.

Gold Nanorod Overgrowth

An overgrowth procedure was implemented to cover any silver that may be at the AuNR surface with gold thus creating a better surface for thiol binding.\textsuperscript{1,4} First, 4 mL of the CTAB coated AuNRs were centrifuged at 10,000 rpm for 20 minutes to remove excess CTAB, the supernatant was removed, and the pelleted AuNRs were resuspended in 4 mL of nanopure water. The AuNR solution was centrifuged a second time and the pelleted AuNRs were resuspended in a sufficient amount of 0.01 M CTAB to reach a AuNR concentration of 900 pM. The
concentration of the AuNR solution was approximated by using the UV-VIS extinction spectrum and the extinction coefficient of $4.4 \times 10^9 \text{ M}^{-1}\text{cm}^{-1}$. Next, sufficient amounts of ascorbic acid (taken from a 100 mM stock solution) and HAuCl$_4$ (taken from a 1 mM stock solution) was added to bring the solution to 1 mM and 0.005 mM respectively. The resulting solution was vortexed and allowed to sit at room temperature for 1 hour. Finally, CTAB was added to bring its concentration to 0.05 M in the solution.

2.2 FUNCTIONALIZATION OF GOLD NANOORDS WITH FLUOROPHORE-LABELLED DNA

The fluorophore-labeled dsDNA used in these experiments were prepared with a thiol on one end, to bind to the AuNR surface, and a reporter fluorescent molecule on the opposite end. The reporter molecule used in the experiments discussed in Chapters 3, 4, and 5 is carboxytetramethyl rhodamine (TAMRA). A second reporter molecule, Atto 532, is also discussed in Chapter 5. Functionalizing the overgrown AuNRs was completed in two major steps.$^{1,4,5}$ The first step consisted of hybridizing single-stranded DNA (ssDNA) to make the double-stranded DNA (dsDNA) used for the nanorod functionalization. Several ssDNAs were used in the experiments discussed in this dissertation, the strands will be referred to as TAMRA ssDNA (3’-[TAMRA]TTCTTAAATATTCGTCTTTTTTTTTTTTTTT-5’), Atto 532 ssDNA (3’-[Atto 532]TTCTTAAATATTCGTCTTTTTTTTTTTTTTTTTT-5’), unlabeled ssDNA (3’TCTTTAATATTCGTCTTTTTTTTTTTTTTTTT-5’), and thiolated ssDNA (5’-AAGAATTTATAAGCAGAAAAAAAAAAA[thiol]-3’). The single strands were purchased from Integrated DNA technologies and it is important to note that the thiolated ssDNA is prepared with a dithiol attached. In Chapters 3, 4, and 5 the TAMRA ssDNA and thiolated ssDNA were hybridized to create the fluorophore-labeled dsDNA for experiments. Chapter 5 also uses Atto 532 as the fluorophore on the labeled dsDNA (Atto 532 ssDNA + thiolated ssDNA). In chapter 3, the thiolated ssDNA was added in excess to act as a spacer to avoid the self-quenching of nearby TAMRA molecules. However, the procedure in Chapters 4 and 5 were changed to use unlabeled dsDNA (unlabeled ssDNA + thiolated ssDNA) as the spacer to avoid
fluorophore self-quenching as well as to provide better structural support for the fluorophore-labeled dsDNA. Figure 2.1 includes cartoons of both procedures that were used to create the functionalized AuNRs.

Figure 2.1  (A) Cartoon depiction of the procedure used to functionalize AuNRs with labeled dsDNA and excess thiolated ssDNA for experiments in Chapter 3. (B) Cartoon depiction of the procedure used to functionalize AuNRs with labeled dsDNA and unlabeled dsDNA in Chapters 4 and 5 experiments.

In Figure 2.1A, the TAMRA ssDNA was mixed with excess thiolated ssDNA in an Eppendorf tube to reach a volume of 100 µL (the concentrations for each can be found in Chapter 3). In Figure 2.1B, the labeled dsDNA, created by mixing TAMRA or Atto 532 ssDNA with the thiolated ssDNA, was hybridized separately from the unlabeled dsDNA (unlabeled ssDNA + thiolated ssDNA). The dsDNA were mixed to reach a volume of 50 µL in each Eppendorf tube, the concentrations of labeled and unlabeled dsDNA used for these experiments can be found in Chapters 4 and 5.

Regardless of which ssDNAs were being mixed, the next step was placing the Eppendorf tube(s) containing the DNA mixtures in a 95°C water bath for 2 minutes and then allowing them to cool down to room temperature for 1 hour. After the cooling period, dithiothreitol (DTT) was added to obtain a concentration of 100 mM in the dsDNA solution. The DTT serves to break the
dithiol attached to the thiolated DNA. The solution was allowed to sit for 30 minutes. The DNA mixtures were then purified via filtration using centri-spin columns (purchased from Princeton Separations) to remove the DTT. Simultaneously, 100 µL of the overgrown AuNR were centrifuged at 10,000 rpm for 20 minutes, the supernatant was discarded, and the AuNRs were resuspended in 100 µL of nanopure water. The overgrown rods were then centrifuged a second time, the supernatant was discarded, and the AuNRs were resuspended in 100 µL of the purified DNA solution. This solution was allowed to sit at room temperature for 1 hour. Finally, 1% sodium dodecyl sulfate (SDS) and 0.1 M phosphate buffer at pH 7 were added to achieve concentrations of 0.01% and 0.01 M respectively.

The second step for functionalizing AuNRs involved gradually increasing the sodium chloride (NaCl) concentration to promote electrostatic screening between the DNA to prevent the dsDNA from bending or swaying to interact with each other. Ideally, this can lead to denser packing of the DNA on the AuNR surface, as depicted in Figure 2.1A and B. Varying amounts of 1 M NaCl (6 µL, 6 µL, 12 µL, 14 µL, 15 µL, and 15 µL) were added in 30 minutes intervals and after each NaCl addition the solution was vortexed and sonicated briefly. After the last sonication the functionalized AuNR solution was allowed to sit at room temperature overnight to allow the reaction to come to completion. Finally, the solution was centrifuged 3 times at 10,000 rpm for 20 minutes, each time the supernatant was removed and AuNRs were resuspended in 100 µL of 0.01% SDS. The solution was then placed in the refrigerator for storage. This AuNR functionalization procedure was only used in experiments discussed in Chapters 3, 4, and 5.

2.3 COVERSILIP PREPARATION

25 x 25 mm #1 thickness glass coverslips were used for all experiments. For the experiments discussed in Chapter 3, three coverslip preparation techniques were used. For the first technique, the coverslips were cleaned using a 3:1 sulfuric acid: hydrogen peroxide soak for 1 hour, rinsed with nanopure water, and dried under nitrogen. Next, 5 µL of the functionalized AuNR solution and 5 µL of a diluted sky blue fluorescent bead solution (1:50 dilution in
nanopure water, beads were purchased from Spherotech) were dropcast onto the coverslip. After 5 minutes, excess functionalized AuNRs and sky blue fluorescent beads that did not adhere to the coverslip, were rinsed off with nanopure water and the coverslip was dried under nitrogen. The sky blue fluorescent beads were used as alignment markers to track and account for mechanical drift over the course of data collection and they were used in all the experiments discussed in this dissertation.6

The second and third coverslip preparation techniques were altered to allow for optical experimentation as well as structure correlation. For both techniques, an aluminum alpha-numerical grid was deposited, via shadow deposition, onto the glass coverslips.7 In both techniques, the gridded glass coverslips were cleaned in an argon plasma (Harrack plasma cleaner) for 15 minutes. Specific to the second technique, the cleaned coverslip was placed in a 0.5% (3-Aminopropyl)triethoxysilane (APTES) in ethanol solution for two minutes while stirring, after which the gridded coverslip was rinsed with ethanol and nanopure water and dried under nitrogen. Next, 5 µL of diluted (1:100 dilution in 0.01% SDS) functionalized AuNRs were deposited onto the gridded coverslip, after 1 minutes the coverslip was rinsed with nanopure water and dried under nitrogen. Then 5 µL of the diluted sky blue beads were deposited, after 15 seconds the coverslip was rinsed and dried under nitrogen.

It was assumed that the addition of the APTES silane layer was a necessity for preventing movement of the AuNR on the coverslip during structure correlation experiments; however, this was not the case. Therefore, the third coverslip preparation technique was also used and it involved only cleaning the gridded coverslips with argon plasma. Next, 5 µL of the 1:100 diluted functionalized AuNR were deposited, after 5 minutes the slide was rinsed with nanopure water and dried under nitrogen. Then 5 µL of the diluted sky blue beads were deposited, after 1 minute the slide was rinsed and dried under nitrogen.

The experiments discussed in Chapter 4 and 5 used a similar approach to the second coverslip preparation technique that was used in Chapter 3 involving the APTES coating. Several changes were made, such as the gridded coverslips were rinsed with ethanol and water and dried
with nitrogen after the 15 minute argon plasma cleaning and prior to placing the coverslip into the 0.5% APTES solution. Also, the functionalized AuNR dilution was changed to 20:100 in 0.01% SDS and the 5 µL diluted sky blue bead solution was allowed to sit for 45 seconds. Chapter 5 also discusses results of experiments conducted on argon plasma cleaned coverslips without the alpha-numerical grid. In this instance, 5 µl of the 20:100 diluted functionalized AuNR solution was deposited on the cover for 5 minutes, after the excess was rinsed off with nanopure water and the coverslip was dried under nitrogen. Next, 5 µL of the diluted sky blue bead solution were dropped and allowed to sit for 5 minutes, the same rinsing and drying protocol followed.

In Chapter 6, 2 mL of CTAB coated AuNRs were first centrifuged twice at 10,000 rpm for 20 minutes, each time the supernatant was discarded and the pelleted AuNRs were resuspended in 2 mL of nanopure water. The gridded coverslip was cleaned with argon plasma for 15 minutes, rinsed with ethanol and water, and then dried under nitrogen. Finally, 5 µL of the rinsed AuNRs were deposited, the coverslip was then rinsed with nanopure water and dried under nitrogen.

2.4 MICROSCOPY

Fluorescence Microscopy

Figure 2.2 shows the experimental setup used for all the fluorescence data collection. The experiments were conducted using an Olympus IX-71 inverted microscope and an Olympus oil-immersion 100x objective with an internal iris to achieve a numerical aperture (NA) between 0.6-1.3. The majority of the experiments discussed in this dissertation used the same 100x objectives, however, data in Chapter 6 was taken with several objectives to decide which are best for super-resolution experiments involving super-localization. For the super-resolution experiments, a NA of 1.3 was used and the samples were imaged via epi-illumination. The 532 nm continuous wave laser excitation source was passed through a quarter wave plate (QWP), to achieve quasi-circularly polarized light, and a lens at the back of the microscope, to achieve wide
field illumination at the sample plane. The excitation source was then reflected off a 532 nm dichroic beam splitter and through the objective to be incident upon the sample. The laser intensity at the sample plane was ~29 kW/cm². Emission signal from the sample was collected back through the objective and passed through a 532 nm long pass filter before being imagined on an electron-multiplied CCD (Princeton Instruments, PhotonMax or ProEM). The data discussed in Chapter 3 was taken using 33 msec/frame integration time, each frame contained 512 x 512 pixels. Multiple movies, each with 1,875 frames were taken for each of the functionalized AuNRs imaged. The data in Chapter 4 and 5 were taken using 33 ms/frame integration time, each frame contained 356 x 356 pixels and 3,650 frames were taken per movie. The experiments in Chapter 6 were taken with an integration time of 0.3 seconds, each frame was 512 x 512 pixels. All optical microscopy in Chapters 4, 5, and 6 took place under nitrogen by covering the sample with a home-built nitrogen flow chamber and imaging under a continuous nitrogen flow.

Figure 2.2  Block diagram of fluorescence microscopy setup.
Dark field Microscopy

Dark field microscopy was used to obtain the scattering spectra for individual AuNRs, as shown in Figure 2.3. The NA on the 100x objective was changed to 0.6. The built-in overhead halogen white light source was passed through a dark field condenser (Olympus, U-DCD 2038101 with a NA of 0.92-0.8), such that high angle light was incident upon the sample. Low angle scattering from a single AuNR was collected using a liquid nitrogen cooled spectrometer (Acton Research Corporation SectraPro-150 or Princeton Instruments 2500i) and camera (Princeton Instruments, Spec-10). For the experiments conducted in Chapter 3, the spectra were taken with an integration of 15 seconds with 2 accumulations. The scattering anisotropy measurements taken for Chapter 3 were created by placing a linear polarizer in the collection path and monitoring the scattering intensity from the AuNR as the linear polarizer was rotated. The experiments conducted in Chapter 4, 5, and 6 were done using an integration time of 60 seconds with 3 accumulations or 60 seconds with no accumulation, depending on which spectrometer/CCD camera system was being used. All dark field scattering spectra were normalized against the halogen lamp profile. Dark field images of the single AuNRs were also obtained by using the electron-multiplied CCD camera with an integration time of 0.3 seconds or 0.2 seconds depending on which CCD camera was being used.
Correlated Atomic Force Microscopy

The correlated atomic force microscopy (AFM) images of the underlying AuNRs used in the optical experiments were also obtained.\textsuperscript{9} The alpha-numerical grid on the coverslip allowed for easy registration of the AuNRs between the optical microscope and AFM. To obtain the structural information, a combined NT-MDT NTegra Vita AFM/total-internal reflection microscope was used.\textsuperscript{9,10}

2.5 Data Processing

In-house written MATLAB code was used to process the data collected.\textsuperscript{1,11} TIFF stacks were created for the movies taken during the experimentation (the size of the stacks differs depending on the experiment). The first step in the analysis was selecting the region around the
functionalized AuNR that was imaged and then selecting a background region to be subtracted away. Next, an integrated intensity vs time trace is created for the background subtracted functionalized AuNR region. Subsequent frames are then subtracted from each other to figure out which frames contain AuNR luminescence only (Frames$_{AuNR}$) and which frames contain AuNR luminescence and fluorescence signal from the reporter molecule (Frames$_{combined}$). Figure 2.4 shows an example of an integrated intensity vs time trace, the green dots mark Frames$_{AuNR}$ and the red dots mark Frames$_{combined}$. In order to be marked with a red dot, the intensity of the Frames$_{combined}$ must be at least 5 standard deviations higher than the average AuNR luminescence intensity. Several frames cannot be clearly identified as red or green and is therefore left unmarked and not included in the analysis.

![Figure 2.4](image)

**Figure 2.4** Example of an integrated intensity vs time trace. The AuNR luminescence only frames are marked with green dots and red dots mark fluorescence signal and AuNR luminescence.

Once the Frames$_{AuNR}$ are identified those frames are fit to either a two-dimensional (2D) Gaussian, Equation 1, (used in Chapters 3, 4, and 6) or another modeling technique which will be referred to as the 3-dipole code that does not have a closed form expression (used in Chapter 4, 5 and 6).
\[ I(x,y) = z_0 + I_0 e^{-\frac{1}{2} \left( \frac{(x-x_0)^2}{s_x} + \frac{(y-y_0)^2}{s_y} \right)} \]  

(1)

In Equation 1, \( I(x,y) \) is the spatially-dependent intensity, \( z_0 \) is the background intensity, \( I_0 \) is the peak intensity, \( s_x \) and \( s_y \) are the widths of the Gaussian in \( x \) and \( y \), and \( x_0 \) and \( y_0 \) is the centroid position.\(^1,8,11\) The other modeling technique, 3-dipole, fits the AuNR luminescence as the sum of three mutually orthogonal emitting dipole modes at an interface and is discussed in Chapter 4.\(^8,11\) With both techniques, the individual Frames\(_\text{AuNR} \) are fit to extract the centroid positions and intensities. These data are averaged to find the average AuNR luminescence contribution. This contribution is then subtracted from frames that have been identified as Frames\(_\text{combined} \). The remaining fluorescence emission is then fit to Equation 1 to extract the centroid position; a goodness of fit parameter (\( R^2 \)) of at least 0.8 is needed to decide if the fit was good. This process is repeated for all the movies taken for each functionalized AuNR resulting in text files containing centroid positions of each identified report molecule. These points can be plotted in a scatter plot to show the position of each reporter molecule that marks the apparent position of labeled dsDNA on the AuNR surface. Due to the data size of the each movie they are processed separately and the average luminescence position was used as a reference point to overlay the centroid positions in the scatter plots from each movie.

2.6 Acknowledgements

I would like to acknowledge my advisor, Dr. Katherine Willets, and labmate, Dr. Eric Titus, for writing the majority of the in-house MATLAB code that was used for data processing in these experiments. I would like to thank Dr. Eric Titus for taking the AFM structure images. I would also like to thank Dr. Kathryn Mayer for being an integral part of getting the sample preparation protocol functioning.

2.7 References


Chapter 3: Triplet-State Mediated Super-Resolution Imaging of Fluorophore-Labeled Gold Nanorods¹

3.1 INTRODUCTION

In previous work, as a proof of concept, we successfully used a triplet-state mediated, ground state depletion followed by individual molecule return (GSDIM),¹ ² super-resolution imaging technique to overcome the diffraction limit and probe binding of fluorophore-labeled double-stranded DNA (dsDNA) on the surface of gold nanowires.³ The length (~1.9 μm) of the nanowire substrates used in those experiments was larger than the diffraction-limited resolution of the microscope and allowed us to qualitatively compare the reconstructed nanowire shape and orientation to the experimental values, which we could directly observe by eye. However, the gold nanorods (AuNRs) discussed in this dissertation are sub-diffraction-limited in all dimensions adding an extra level of difficulty to this experiment. The work in Chapter 3 focuses on whether we can both reconstruct the size, shape, and orientation of a single AuNR as well as determine the sites of ligand binding on its surface using GSDIM from fluorophore-labeled dsDNA attached to the AuNR surface.

Previous super-resolution work on AuNRs has used catalytically-generated fluorescence to reconstruct the shape of the nanorod.⁴ In those studies, a single non-fluorescent Amplex Red molecule diffused to the nanorod surface, was converted to its fluorescent form, and then the emission was fit to a 2-Dimensional (2-D) Gaussian. The shape of the nanorod was reconstructed by waiting for enough fluorescence turnover events to completely map the nanorod surface. By controlling the concentration of the Amplex Red, the authors could ensure only a single emissive molecule was present within the diffraction-limited spot at a time. In our studies, we functionalize the surface of the AuNR with hundreds of dye molecules, which means we need

to control the emissive states of many molecules co-localized within a single region of interest in order to reconstruct the shape of the underlying nanorod substrate.

We also test the performance of the 2-D Gaussian model for fitting the diffraction-limited emission from both the ligands and the inherent AuNR luminescence to successfully reconstruct the AuNR properties. We have recently shown that a 2-D Gaussian introduces localization errors when fitting AuNR luminescence, especially for AuNRs with an out-of-plane tilt. However, more accurate models based on dipolar emission are computationally expensive and most super-resolution work favors the 2-D Gaussian model, despite its limitations. Here, we show that despite the localization errors inherent to the model, the 2-D Gaussian performs quite well for mapping AuNR shape and orientation and providing insight into ligand binding on the surface.

3.2 IMAGING FLUORESCENTLY-LABELED AuNRs

For these studies, AuNRs (either 99 ± 8 nm long x 39 ± 3 nm wide or 81 ± 7 nm long x 29 ± 3 nm wide, depending on the sample, Figure 3.1) were functionalized with dsDNA, with a thiol group at one end (for binding to the nanorod) and a fluorescent dye molecule at the other end. The fluorescent molecule used in this study is carboxytetramethyl rhodamine, or TAMRA. During the functionalization process 50 µL 14.4 µM of thiolated single-strand (ssDNA) and 50 µL of 3.6 µM TAMRA ssDNA were hybridized together in an Eppendorf tube, leaving thiolated ssDNA in excess. This ratio of thiolated ssDNA to TAMRA ssDNA was chosen so that the excess thiolated strands would provide spacing between the TAMRA molecules on the nanorod surface. TAMRA molecules too close together will undergo self-quenching and be non-fluorescent. The AuNR synthesis protocol and the functionalization process are discussed in detail in Chapter 2. While the presence of a dye molecule may impact the DNA binding to the surface (as compared to its unlabeled counterpart), the dye provides us with a readout of the location of the labeled ligand, which is critical to the success of these studies.
Figure 3.1  STEM images of representative AuNRs from the two different solutions used in the experiments described in the main paper. AuNRs in (A) are representative of the functionalized AuNR data presented in Figure 3.4. AuNRs in (B) are representative of the functionalized AuNR data shown in Figures 3.6-9. Scale bars equal 50 nm.

Figure 3.2  Dark field scattering spectra for the AuNRs discussed in Figures 3.6-3.9. The spectra are ordered by when they are first discussed in this Chapter (e.g. (A) corresponds to the AuNR discussed in Figure 3.6). The peak of highest scattering intensity for the AuNRs and TAMRA emission (~580 nm) do not strongly overlap.
TAMRA has a peak emission of ~580 nm, can be excited with 532 nm laser excitation, and readily undergoes intersystem crossing. Importantly, the emission spectrum of TAMRA does not substantially overlap with the localized surface plasmon resonance of the AuNRs used in this study ($\lambda_{\text{max}}$ between 630 and 670 nm, Figure 3.2). In our previous super-resolution studies on gold nanowires, we found that TAMRA emission could couple into the surface plasmon polariton modes of the nanowire, such that the location of the emission was not necessarily correlated with the binding site of the fluorophore label. Because we are interested in studying site-specific ligand binding in this study, we want to minimize coupling between the TAMRA fluorescence and the AuNR plasmon, which is achieved by minimizing their spectral overlap. Moreover, fluorescent molecules that are too close to a plasmonic surface will experience quenching. The dsDNA linker used here consists of 28 base pairs, which provides a distance of ~9.52 nm (0.34 nm/base pair) between the TAMRA and the AuNR surface. The dsDNA spacer puts the TAMRA molecules outside the quenching regime of ~4.8 nm away from the AuNR surface.
Figure 3.3  (A) Integrated intensity versus time from a representative TAMRA-labeled AuNR. The inset shows a zoomed-in portion of the time trace to highlight the on/off behavior of the GSDIM-controlled emission. (B) Integrated intensity versus time trace from the same AuNR sample as (A) ~11 minutes later. The inset shows that the ideal GSDIM switching behavior is only controlled for a limited amount of time.

Functionalized AuNR samples are dropcast from solution onto glass coverslips and imaged under nitrogen with 532 nm excitation onto a CCD camera. Individual nanorods are observed as diffraction-limited spots within the field of view that show distinct intensity fluctuations associated with GSDIM-mediated photoswitching. Figure 3.3A shows a representative intensity time trace, in which fluorescent bursts from individual TAMRA dyes are observed above a constant AuNR luminescence background. Figure 3.3B shows a time trace from the same AuNR, but ~11 minutes later in the data acquisition. We note that the AuNR luminescence signal has increased in this later movie, and that the on/off switching behavior of the TAMRA molecules is less pronounced. The reason for the increase in the AuNR
luminescence could either be (1) light-mediated surface restructuring of the AuNR generating enhanced luminescence or (2) an increased background from TAMRA molecules that are not efficiently remaining in the dark triplet state. We hypothesize that the second mechanism is most likely responsible for the increased background, given the reduced contrast in the photoswitching, although the reason for this change in photophysical behavior is unclear. One possibility is that the DNA is unstable on the surface due to plasmon-induced local heating, leading to a collapse or local rearrangement of the DNA and a change in the associated TAMRA photophysics. Because we observe a gradual deterioration in the photoswitching behavior of the TAMRA across all of our samples, our data collection period is limited. Despite this challenge, we find that the data collected before the photophysics change can reliably and reproducibly be used to reconstruct the image of the underlying AuNRs.

3.3 RECONSTRUCTED IMAGES OF FUNCTIONALIZED AuNRs

From the time trace, we assign each image as either AuNR luminescence or a combination of both AuNR luminescence and TAMRA emission (see Chapter 2 for more details on image processing and analysis). Images associated with AuNR luminescence alone are each fit to a 2-D Gaussian, as shown in equation 1:

$$I(x,y) = z_0 + I_o e^{-\frac{1}{2}\left[\frac{(x-x_0)^2}{s_x^2} + \frac{(y-y_0)^2}{s_y^2}\right]}$$

(1)

Here I is the spatially-dependent intensity of the diffraction-limited emission spot, z₀ is the intensity of the background, I₀ is the peak intensity of the emission, sₓ and sᵧ are the widths of the Gaussian in x and y, and x₀ and y₀ are the centroid position of the peak intensity, which is assumed to reflect the location of the emitter. From the fits to the AuNR luminescence, we calculate an average 2-D Gaussian that models the contribution of the AuNR luminescence. Next, for each image associated with TAMRA emission, we subtract the 2-D Gaussian model of
the AuNR luminescence contribution, in order to leave only the contribution of the TAMRA emission behind. The remaining TAMRA emission is then fit to equation 1, and the centroid position of the fluorescent emitter is determined for each image. The result is a scatter plot where we have both the centroid positions of the isolated TAMRA emitters and the average centroid position of the AuNR luminescence.

Figure 3.4 shows the first super-resolution imaging example of a TAMRA-labeled AuNR. The dark-field scattering spectrum of the AuNR is shown in Figure 3.4A, with a longitudinal localized surface plasmon resonance peak maximum of ~639 nm. This maximum is far from our excitation wavelength of 532 nm and shows minimal overlap with the TAMRA emission maximum at ~580 nm, suggesting that we would not expect to see surface-enhanced fluorescence due to local electromagnetic field enhancements or coupling between the fluorescence emission and the plasmon. To determine the orientation of the nanorod (and confirm that we have a single nanorod), we inserted a linear polarizer in front of our spectrometer and rotated the angle in 20º increments in order to measure the scattering anisotropy. The resulting polar plot showing the scattering intensity against polarization angle is given in Figure 3.4B and shows the expected dipole-like behavior from a single nanorod, while also indicating the orientation of the nanorod on the surface of the coverslip. The anisotropy data were fit to equation 2, where \( f \) is the scattering intensity from the AuNR when the linear polarizer is at angle \( \theta \), \( I \) is the peak intensity, \( \varphi \) is the in-plane orientation angle of the nanorod, and \( c \) is an offset. Fitting the data from Figure 3.4B to this equation yields an in-plane orientation angle (\( \varphi \)) of 54.5º.

\[
f(\theta) = I \cdot \cos^2(\theta - \varphi) + c \tag{2}
\]
Figure 3.4  (A) Dark-field scattering spectrum of a functionalized AuNR. The excitation wavelength and TAMRA emission maximum are indicated to show there is minimal overlap with the plasmon resonance. (B) Polar plot of AuNR scattering as a function of emission polarization angle. Best fit to data shows AuNR is oriented at 54.5 degrees. (C) Scatter plot displaying the centroid positions of 479 unique TAMRA emission events (blue dots) as well as the average luminescence centroid (red x). (D) Frequency histogram showing the frequency of TAMRA emission events as a function of position. (E) Spatial intensity map showing the average intensity of the TAMRA emission from all points within each bin of the frequency histogram. The bin size for (D) and (E) is 4.34 nm. (C) (D) and (E) have equal 50 nm scale bars.

Next, GSDIM data was acquired and fit according to the procedure outlined above. The scatter plot in Figure 3.4C depicts the centroid positions of unique TAMRA emission events that
were fit, shown as blue circles. The blue data points show the expected rod-like structure, oriented in the same direction as the scattering anisotropy, indicating that we have successfully reconstructed the expected shape and orientation of the AuNR with our GSDIM approach. To create this scatter plot, 479 TAMRA emission events were localized and plotted from 28,110 total frames of collected data. Based on the dimensions of the AuNR, we expect ~768 DNA molecules to be bound to the surface, assuming monolayer coverage.\textsuperscript{8} In our sample preparation, we introduce the thiolated ssDNA in a 4:1 excess of the complementary TAMRA-labeled ssDNA; thus we expect to have a mixture of unlabeled single stranded and TAMRA-labeled dsDNA on the AuNR surface. Assuming the original solution stoichiometry is preserved on the surface, this suggests that we should have ~154 TAMRA labels bound to the AuNR. Given that we observe 479 distinct emission events, it appears that the TAMRA molecules are being sampled multiple times to create the scatter plot in Figure 3.4C.

The red “x” on the scatter plot represents the average position of the nanorod luminescence with a standard deviation of 3 nm and 4 nm, in the x- and y-dimensions, respectively. Notably, the red “x” is slightly off-center from the blue data points in the scatter plot. We would expect the luminescence centroid to be centered with respect to the TAMRA emission events, assuming that the luminescence centroid is associated with the center of the AuNR. As discussed previously, using a 2-D Gaussian model to fit AuNR luminescence data can introduce localization inaccuracies in the position of the AuNR luminescence centroid, particularly if the AuNR is oriented slightly out-of-plane; this could be the source of the slight offset in the AuNR luminescence centroid. On the other hand, most AuNRs are not perfectly symmetric, and the expectation that the luminescence centroid should be exactly centered on the nanorod may be an imperfect assumption, which could also explain the offset between the center of the blue data and the position of the luminescence centroid.

To further analyze the data, the TAMRA emission events were binned into 4.34 nm bins (corresponding to 1/10\textsuperscript{th} of an imaging pixel) to create the frequency histogram shown in Figure 3.4D. This bin size is consistent with the standard deviation in the AuNR luminescence centroid.
and represents a lower bound of our expected precision. The color of each bin indicates the number of TAMRA emission events that occurred within the location defined by the bin. Again, given our calculation of ~154 dye labels per nanorod and our observation of 479 distinct emission events, the frequency histogram does not reflect the number of bound molecules, but rather how often emission events are observed at a particular location. The frequency histogram shows that the TAMRA emission events are biased towards the upper end of the nanorod, rather than uniformly distributed along the nanorod surface. One possibility is that the labeled DNA binds preferentially to one end of the nanorod over the other, assuming that the photoswitching probability of each dye molecule is spatially invariant (e.g. 479/154 \approx 3 \text{ emission events per TAMRA}). Preferential ligand binding at the nanorod ends has been noted by several other groups, although there is no evidence suggesting that one end should be biased over another.\textsuperscript{13,14} However, if cooperative binding occurs, such that binding of one type of DNA (unlabeled single stranded or labeled double stranded) leads to increased local binding of that same type, then we might expect to observe asymmetric binding in our frequency histograms.\textsuperscript{15,16} A second possibility for the apparent binding asymmetry is that the fluorescently-labeled ligands on one end of the nanorod were excited more efficiently than the ligands on the other end, leading to a higher frequency of TAMRA emission events at the one end. One possible explanation for this theory is that a slight out-of-plane tilt could lead to asymmetric ligand excitation; however, later examples will refute this theory. A second explanation is that we have plasmon-enhanced fields at the nanorod ends, which generate more contrast in the GSDIM photoswitching. Link and coworkers have shown that exciting the transverse plasmon mode in AuNRs can generate a hot electron, which can be transformed into a lower energy plasmon, resonant with the longitudinal mode.\textsuperscript{17} If this were the case, then we would expect to see higher fluorescence intensity from the TAMRA emission events at the nanorod ends, due to the locally enhanced electromagnetic fields.

To probe the possibility that plasmon-enhanced local electromagnetic field enhancements affect the local TAMRA photophysics, we constructed a spatial intensity map, as shown in
Figure 3.4E, in which we calculate the average intensity of all TAMRA emission events within each bin of the frequency histogram. If plasmon-enhanced electromagnetic fields were present, we would expect to observe higher fluorescence intensity from TAMRA molecules located in these regions of local electromagnetic field enhancement. The spatial intensity map shows that the fluorescence intensity along the nanorod is evenly distributed, varying only by a factor of ~2. While the highest intensity bins are located at the nanorod ends, they do not correspond to the high frequency bins in the frequency histogram. Moreover, if we did have regions of higher intensity, we would expect them to be spread over multiple bins, rather than localized on a single bin. Because the average intensity per bin is plotted in the spatial intensity map, a single occurrence of a high intensity burst will skew the results. Given that we do not see a direct correlation between high frequency and high intensity bins, it is unlikely that the apparent asymmetric binding observed in the frequency histogram can be explained by plasmon-enhanced electromagnetic fields at the nanorod end leading to increased TAMRA emission events. Thus, the most likely explanation is that the TAMRA-labeled DNA is preferentially bound to one end of the AuNR.

According to the scanning transmission electron microscopy (STEM) images of the nanorod solution used for this example (Figure 3.1A), the reconstructed image should have dimensions of 99±8 nm (length) x 39±3 nm (width). The reconstructed nanorod in Figure 3.4 is approximately 60 nm long and 20 nm wide, well below the expected dimensions of the actual nanorod. We hypothesize that because a 2-D Gaussian does not completely represent the point spread function of the nanorod luminescence, we are not successfully subtracting off the entire contribution of the AuNR from the images corresponding to TAMRA emission during the data analysis. By failing to subtract away the complete AuNR contribution, the TAMRA emission events become biased towards the luminescence centroid location, collapsing the dimensions of the nanorod. We will test this hypothesis in Chapter 4, in which we compare the fits obtained using a 2-D Gaussian model to a more rigorous dipole-based model.
3.4 Reconstructed Functionalized AuNR Images with Structure Correlation

To better understand the difference between the expected and reconstructed dimensions of the AuNR, we performed structure correlation experiments. In previous work, we used indium tin oxide (ITO) coated coverslips as substrates because they are both optically transparent (for imaging experiments) and conductive (for electron microscopy).\textsuperscript{3,18} Unfortunately, the presence of the ITO substrate perturbed the photophysics of the TAMRA, such that we no longer observed clear photoswitching behavior (Figure 3.5). Attempts to improve the GSDIM-based photoswitching by changing environment (mixing O\textsubscript{2} and N\textsubscript{2}) and adjusting laser intensity yielded no success. Because of this, we turned to atomic force microscopy (AFM) to determine AuNR structure, although we note that tip effects will convolve the shape of the AFM tip with the actual AuNR structure. Nonetheless, this technique is compatible with our glass coverslip substrate, and allows us to compare AuNR orientation and shape with our reconstructed images.\textsuperscript{19}

![Figure 3.5](image1.png)

(A) Integrated intensity versus time from a representative TAMRA-labeled AuNR on an ITO coated coverslip. The inset shows a zoomed-in portion of the time trace to display that the ideal GSDIM switching behavior is unattainable on ITO.
Figure 3.6 shows an example of correlated GSDIM and AFM imaging. As before, we construct a frequency histogram (Figure 3.6A) and spatial intensity map (Figure 3.6B) from the centroid data, with a white “x” showing the location of the AuNR luminescence. The frequency histogram, Figure 3.6A, shows a higher occurrence of TAMRA emission events at the ends of the nanorods. This supports the hypothesis that ligands may bind preferentially, but not exclusively, to the end of AuNRs. 573 TAMRA emission events, occurring over 31,858 frames, were localized in this example. The spatial intensity map, Figure 3.6B, shows fairly even intensity values distributed over the nanorod, with roughly a factor of two difference in intensity across the nanorod. As observed previously, the average AuNR luminescence centroid position is slightly off-center from the reconstructed image of the rod.

Figure 3.6C shows the AFM image of the AuNR, with the frequency histogram from Figure 3.6A laid on top of the nanorod with a common 50 nm scale bar. The overlaid image shows that the orientation between the AFM image and the reconstructed image agree. However, we once again note that the dimensions of the reconstructed image are well below
what is expected, based on both the AFM data shown here and associated STEM data (which predicts an average length and width of 81±7 nm and 29±3 nm, respectively, Figure 3.1B). Without accounting for tip effects, the AFM image predicts that this AuNR is 153 nm x 114 nm. However, based on the height of the AuNR (33 nm) and our expectation that the rod should be roughly cylindrical in shape (such that the width ≈ height), we see that tip effects lead to an overestimation of the width dimension by 81 nm. Using this value for a quick back-of-the-envelope calculation, we can correct the dimensions of the AuNR, reducing it to 72 nm x 33 nm. Even with this correction, the reconstructed image has smaller dimensions than expected, yielding length and width values of approximately 50 nm and 20 nm, respectively. We expect that even if the photophysics of the dye allowed us to use electron microscopy to characterize the nanorod dimensions, the reconstructed images would still be below the actual nanorod size, based on the average nanorod dimensions as determined by STEM. The aspect ratio of the AuNR agrees between the AFM image, 3.6C, and the reconstructed image, 3.6A. Reconstructing smaller than expected dimensions is a recurring trend throughout all the examples shown in this chapter.
Figure 3.7 Examples of uniform GSDIM occurring across the nanorod surface. (A) Frequency histogram and (B) spatial intensity map created by binning 632 significant fluorescent bursts. (C) AFM image showing the structure and orientation of the nanorod reconstructed in (A) and (B). The height of this nanorod is 31 nm. (D) Frequency histogram and (E) spatial intensity map created by binning 1,537 significant fluorescent bursts. (F) AFM image showing the structure and orientation of the nanorod reconstructed in (D) and (E). The height of this nanorod is 33 nm. The bin size for (A-B) and (D-E) is 4.34 nm. All scale bars are 50 nm. The height scale bars for (C) and (F) are in nm. The white “x” in (B) and (E) represents the average luminescence centroid position of the respective nanorod example.

Although the first two AuNR examples have shown a higher frequency of TAMRA emission events at the ends of the nanorod, Figure 3.7 illustrates two examples in which fairly uniform TAMRA emission is observed over the nanorod surface. The frequency histograms, Figures 3.7A and 3.7D, and spatial intensity maps, Figures 3.7B and 3.7E, display that TAMRA emission events occur evenly over the length of the nanorods with fairly constant intensity. The reconstructed images show excellent agreement with the shape and orientation of the AuNR
AFM images, Figures 3.7C and 3.7F, although once again, the reconstructed sizes are smaller than expected. The reconstructed images in Figures 3.7A and 3.7B were created from 632 significant TAMRA emission events collected over 37,480 frames. The average luminescence centroid, the white x, is slightly off center, as seen in previous examples. Figures 3.7D and 3.7E were constructed from 1,537 TAMRA emission events collected over 37,480 frames, indicating that the fluorophore labels on this nanorod were much more active than others discussed so far.

Figure 3.8 Examples of asymmetric TAMRA emission events with different local intensities occurring across the nanorod surface. (A) Frequency histogram and (B) spatial intensity map, created by binning 558 significant fluorescent bursts. (C) AFM image showing the structure and orientation of the nanorod reconstructed in (A) and (B). The height of this nanorod is 30 nm. (D) Frequency histogram and (E) spatial intensity map, created by binning 798 significant fluorescent bursts. (F) AFM image showing the structure and orientation of the nanorod reconstructed in (D) and (E). The height of this nanorod is 34 nm. The bin size for (A-B) and (D-E) is 4.34 nm. All scale bars are 50 nm. The height scale bars for (C) and (F) are in nm. The white “x” in (A) (B) (D) and (E) represents the averaged luminescence centroid position of the respective nanorod examples.
Previously in this chapter, we discussed the possibility that the apparent asymmetric binding of the TAMRA-labeled dsDNA could be linked to regions of enhanced electromagnetic fields. Figure 3.8 shows two examples of reconstructed nanorod images that refute this theory, while also highlighting the heterogeneity inherent to the AuNR substrates. In Figure 3.8A, the frequency histogram shows a higher occurrence of TAMRA emission events on the lower end of the nanorod. The associated spatial intensity map, Figure 3.8B, shows that this same region of the nanorod is associated with higher overall local fluorescence intensity, consistent with the theory that local electromagnetic field enhancements could generate more emission events per TAMRA dye. However, Figure 3.8D also shows a frequency histogram with a bias in TAMRA emission events on the lower end of the AuNR, but in this case, the higher intensity events are associated with the upper end of the nanorod, as shown in the spatial intensity map in Figure 3.8E. Thus, we conclude that regions of higher intensity are not correlated with the increased frequency of TAMRA emission events. In contrast to previous examples, these two AuNRs both have obvious “hot spots,” or regions of high emission intensity, which could potentially be associated with locally enhanced electromagnetic fields produced via hot electron conversion, as described previously. We also find that the luminescence centroids, particularly in Figure 3.8E, appear fairly centered in both examples, indicating that any out-of-plane tilt of the AuNR is most likely not responsible for the apparent asymmetric ligand binding. Thus, we conclude that the dsDNA is most likely binding with different affinity to the different ends of the nanorod. As with previous examples, the reconstructed images agree extremely well with the shape and orientation of the AuNRs, as determined by AFM (Figures 3.8C and 3.8F).

Figure 3.9 provides two final sets of reconstructed images, in which activity at both AuNR ends, with virtually no activity in the center, is observed. The frequency histograms, Figures 3.9A and 3.9D, indicate higher numbers of TAMRA emission events on the ends. The first spatial intensity map, Figure 3.9B, displays evenly distributed intensity across the nanorod. Although the average nanorod luminescence signal is not centered in Figures 3.9A and 3.9B, the technique still provides a good reconstructed image, showing excellent agreement between the
shape and orientation of the nanorod (Figure 3.9C). The second spatial intensity map, Figure 3.9E has a region of higher intensity at the lower left end of the nanorod, spread over multiple bins, once again consistent with a “hot spot.” As with previous examples, the location of the “hot spot” is not correlated with regions of increased TAMRA activity, suggesting that the increased emission intensity is somehow mediated by the nanorod structure itself. Once again, excellent agreement is observed in both the shape and orientation of the nanorod between the reconstructed and AFM (Figure 3.9F) images.

Figure 3.9 Examples showing clear TAMRA emission events localized at the ends of the nanorods. (A) Frequency histogram and (B) spatial intensity map created by binning 303 significant fluorescent bursts. (C) AFM image showing the structure and orientation of the nanorod reconstructed in (A) and (B). The height of this nanorod is 32 nm. (D) Frequency histogram and (E) spatial intensity map, created by binning 680 significant fluorescent bursts. (F) AFM image showing the structure and orientation of the nanorod reconstructed in (D) and (E). The height of this nanorod is 32 nm. The bin size for (A-B) and (D-E) is 4.34 nm. All scale bars equal 50 nm. The height scale bars for (C) and (F) are in nm. The white “x” in (A-B) and (D-E) represents the average luminescence centroid position of the respective nanorod examples.
3.5 **Discussion**

The examples shown here highlight the diverse behaviors of ligand-labeled AuNRs, suggesting that local heterogeneity can play a strong role in dictating both the optical properties of the nanorods as well as ligand binding on their surface. All of the reconstructed images showed a rod-like shape with the correct orientation, when compared to either a scattering anisotropy plot or against a correlated AFM image. Therefore, GSDIM super-resolution imaging of fluorophore-labeled ligands has successfully reproduced many of the structural features associated with the nanorod samples. However, the reconstructed dimensions for all the examples shown in this paper were uniformly smaller than expected. In our previous work on gold nanowires, we found remarkable agreement between the dimensions of the reconstructed super-resolution images and associated electron micrographs; thus, we do not believe this is a flaw in the technique, but rather in the analysis. In the gold nanowire work, we were able to subtract off the luminescence from the nanowire directly rather than model its emission, because of its large size relative to the diffraction-limit, which enabled us to line up the images before subtracting away the nanowire contribution. Here, due to the small size of the AuNRs relative to the diffraction limit, we could not directly visualize the contribution of the AuNR to the overall signal, and instead modeled the AuNR luminescence as a 2-D Gaussian. However, as we have noted previously, this is an imperfect model for fitting the AuNR luminescence data and may lead to insufficient removal of the AuNR luminescence contribution from the overall signal. Consequently, the centroid positions of the TAMRA emission events are skewed towards the luminescence signal, reducing the overall size of the reconstructed nanorod.

In nearly all examples, the average luminescence centroid positions were not centered with respect to the TAMRA emission events. We have hypothesized two possible explanations for this. First, for nanorods that are not perfectly flat on the substrate surface, a 2-D Gaussian will introduce localization inaccuracies, shifting the centroid as many as tens of nanometers away from its true position. Second, the nanorods themselves may have local heterogeneities that are not apparent from AFM or electron microscopy, but lead to a shift of the luminescence signal.
away from the nanorod center. These local heterogeneities are consistent with the observed “hot spots” seen in Figures 3.8 and 3.9. We plan to explore these two possibilities in greater detail in future work, in which we will test more rigorous fitting models on these data to determine if this will improve both the dimensions of the reconstructed images and the position of the luminescence centroid.

From the spatial frequency histograms in Figures 3.4 and 3.6-3.9, we observed several different behaviors in the apparent binding of the TAMRA-labeled dsDNA on the AuNRs: asymmetric binding across the nanorod, uniform binding across the nanorod, and preferential binding to both ends. The locations of higher frequency TAMRA emission events vary between the examples, potentially providing evidence of the heterogeneity in the apparent ligand distribution on gold nanorods. The most important question is whether our reconstructed images genuinely reflect the binding of TAMRA-labeled ligands on the surface of the nanorods. To try and prevent fluorescence-plasmon coupling, we minimized spectral overlap between the two processes, but we also note that several examples showed local “hot spots” which could indicate a plasmon-mediated local electromagnetic field enhancement. We noted, however, that these “hot spots” were not necessarily correlated with regions of increased TAMRA activity, leading us to hypothesize that local binding heterogeneity could be responsible for our results. It is not unexpected that the DNA would bind preferentially to specific sites on the nanorod surface; previous work has noted that certain ligands bind specifically to nanorod ends.13–15,20,21 Moreover, work from Gibbs-Davis and coworkers has shown that the solution-stoichiometry of a mixed ligand system is not necessarily preserved on the surface of a nanoparticle.16 In our case, the mixture of unlabeled ssDNA and TAMRA-labeled dsDNA might lead to cooperative binding on the nanorod surface, in which binding of one ligand type to a particular site leads to preferential local ligand binding of that same type. This cooperative binding behavior could lead to the apparent asymmetry in the ligand binding across the different samples. To further refine our experiment and test our ability to more accurately map the distribution of ligands on the surface, we plan to work at varying ratios of labeled/unlabeled molecules to see if the solution
stoichiometry is preserved on the surface, as well as test mixed samples of dsDNA labeled with different fluorescent dyes. By tuning the stoichiometry and concentration of the DNA labels, we expect to gain better insight into the actual distribution of ligands on the nanorod surface.

Although future work is needed to tease out the answer to the question of apparent vs. actual ligand binding, our results clearly show the heterogeneity intrinsic to ligand-functionalized nanorod samples. Despite the relative uniformity of the AuNRs (Figure 3.1), the optical properties of the attached ligands vary from rod-to-rod, suggesting that subtle differences in local nanorod structure can lead to differences in fluorophore emission, ligand binding, and possibly luminescence centroid location. Thus, single nanoparticle studies that are sensitive to these differences are of critical importance when designing ligand-labeled nanoparticle substrates for bioimaging,\textsuperscript{22–26} theranostic,\textsuperscript{24,27,28} or spectroscopic\textsuperscript{29,30} applications.

3.6 Conclusions

By using GSDIM to study fluorescently-labeled DNA bound to the surface of AuNRs, we have reconstructed the shape and orientation of AuNRs and gained insight into the heterogeneity inherent to ligand-functionalized nanorod samples. We were able to optimize the typical GSDIM fluorescent blinking behavior such that approximately one fluorescent molecule, in a population of hundreds, was emissive at a time within a diffraction limited spot. By fitting the emission to a 2-D Gaussian, we reconstructed the shape and orientation of each AuNR, as confirmed via AFM, although the size of the reconstructed image was consistently smaller than the actual size of the rod. We observed heterogeneity in the apparent binding characteristics of the fluorescently-labeled DNA on the nanorod surface, based on frequency histograms which revealed higher probabilities of TAMRA emission events at different sites on the nanorod surface. In some cases, we found evidence of local “hot spots” based on regions of increased emission intensity in spatial intensity maps. These diverse behaviors are hidden by traditional ensemble measurements, underscoring the importance of single nanoparticle measurements on the molecular (<10 nm) length scale.
3.7 ACKNOWLEDGEMENTS

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3.8 REFERENCES


Chapter 4: Comparing the Accuracy of Reconstructed Image Size in Super-resolution Imaging of Fluorophore-labeled Gold Nanorods Using Different Fit Models

4.1 INTRODUCTION

Recently, super-resolution imaging has emerged as a tool for studying the position of single fluorescent molecules tethered to a wide range of substrates, including the cytoskeleton in eukaryotic cells, cellular membranes, and metallic nanostructures.1–8 In many of these studies, the shapes and sizes of the underlying substrates can be reconstructed, often with high fidelity, by mapping the positions of each fluorescent tag on the substrate surface and building a composite image from the individual emitters. Previously, we have used similar super-resolution fluorescence imaging techniques to map the location of fluorescently-labeled double-stranded DNA (dsDNA) bound to the surface of single AuNRs via a thiol linker.2 While we were able to recover the shape and orientation of the AuNR support in the reconstructed images, we found a consistent under-estimation in the size of the nanorods. Similar disagreements have been observed by Uji-i and coworkers, who reported differences between the actual diameters of silver nanowires and the reconstructed image size produced by super-resolution imaging of fluorescent proteins tethered to the nanowire surface.3,4 In their case, they attributed the size mismatches to distortions of the point spread function (PSF) of the fluorescent emitter, introduced by its proximity to the metallic substrate.3 These PSF aberrations were apparent by eye in their raw data, and showed a dependence on the width of the nanowires studied (110 nm and 250 nm).3 In our previous work, and in the work presented here, we have used both nanowires and nanorods with widths below 100 nm (~68 nm and ~35 nm, respectively) and have seen no visible evidence of PSF distortions in our raw data;1,2 thus we seek an alternative hypothesis to explain the lower-than-expected dimensions of the reconstructed AuNR super-resolution images.

For our super-resolution studies, we use carboxytetramethyl rhodamine (TAMRA) as a fluorescent label attached to dsDNA ligands. TAMRA was chosen because its fluorescence maximum, ~580 nm, does not overlap with the localized surface plasmon resonance (640-690 nm for the functionalized AuNRs discussed in this paper, Figure 4.1) of the AuNRs, based on single particle dark field microscopy scattering spectra. This combination of fluorophore and nanorod was chosen to lower the probability of TAMRA emission coupling into the AuNR which leads to surface enhanced fluorescence\(^9\) and skewed centroid positions of the fluorophore.\(^{10}\) TAMRA molecules can be switched between a fluorescent (on) and non-fluorescent (off) state by promoting intersystem crossing into non-emissive triplet states, a technique known as ground state depletion with individual molecule return (GSDIM).\(^{1,2,7,11}\) DNA is an ideal ligand for these studies because it is thought to assemble into highly ordered monolayers on the nanoparticle surface, spacing the TAMRA molecules a fixed distance from the surface.\(^{12-14}\) Ideally, only one TAMRA molecule is on at a time and its diffraction-limited emission is fit to a 2-dimensional (2-D) Gaussian (equation 1) where \(I(x,y)\) is the spatially-dependent intensity, \(z_0\) is the background intensity, \(I_0\) is the peak intensity, \(s_x\) and \(s_y\) are the widths of the Gaussian in \(x\) and \(y\), and \(x_0\) and \(y_0\) is the centroid position. The location of the single TAMRA emitter is approximated as the centroid position \((x_0, y_0)\). The process is then repeated multiple times, such that the position of each TAMRA molecule is localized, in order to gain a complete picture of where the dsDNA ligands are bound to the AuNR surface.

\[
I(x,y) = z_0 + I_0 e^{-\left[\frac{1}{2}\left(\frac{x-x_0}{s_x}\right)^2 + \left(\frac{y-y_0}{s_y}\right)^2\right]} \tag{1}
\]
Figure 4.1 Dark field scattering spectra for the underlying AuNRs discussed in this chapter. The title of each spectrum details which functionalized AuNR it was taken from.

Unfortunately, the underlying AuNR substrate is inherently luminescent and produces a constant intensity contribution to the diffraction-limited emission. Because the AuNR and TAMRA dyes are spaced by ~10 nm, the two emitters cannot be spatially resolved, according to the Abbe criterion. Failing to account for the presence of the AuNR background leads to a calculated centroid that reflects the intensity-weighted super-position of both the TAMRA and the AuNR emission, generating incorrect information about the position of the dsDNA ligands on the surface. Therefore, we need a method to differentiate between the centroid positions of the two signals: (1) bound TAMRA molecules undergoing photoswitching and (2) steady AuNR luminescence, in order to more accurately determine the position of each ligand on the surface.

In previous work mapping the position of fluorescently-labeled dsDNA attached to a AuNR, we fit the AuNR signal ($I_{AuNR}$) using equation 1, and then subtracted the results of that fit ($I_{AuNR,fit}$) from frames where both TAMRA and the AuNR luminescence were present ($I_{combined}$) to isolate the contribution from the TAMRA alone ($I_{TAMRA} = I_{combined} - I_{AuNR,fit}$). We then fit
ItAMRA to equation 1 to localize the centroid position of the fluorophore and thus the apparent position of the dsDNA ligand. We found significant heterogeneity among different AuNRs with respect to the apparent locations of the dsDNA bound to the surface, which is information that is hidden in a bulk measurement. However, while the images reconstructed from the super-resolution data matched the shape and orientation of the underlying AuNR supports, the size of the reconstructed image was always smaller than the expected dimensions of the AuNRs. If we were mapping the true location of the dsDNA then we would expect the reconstructed images to match the measured dimensions of the AuNR, assuming uniform coverage of dsDNA. We hypothesized that our choice of a 2-D Gaussian for fitting the AuNR luminescence could be influencing the super-resolution results. This model was chosen for its simplicity and because it has been used by the bulk of the super-resolution community, yet it has no physical meaning and can introduce significant aberrations into the fits.

On the other hand, our group has previously shown that AuNR luminescence is well-modeled as the sum of three mutually-orthogonal emitting dipoles at an interface; we will refer to this as the 3-dipole model through the rest of this manuscript. In the 3-dipole model, a dipole corresponding to the longitudinal axis of the AuNR is described by the in-plane angle of the AuNR along its long axis, $\phi$, and the out-of-plane angle of the AuNR, $\theta$, (see Figure 4.2 for angle definitions). Two other mutually orthogonal dipoles are included to model the short axes of the AuNR. The emission from the three dipoles sitting at the air-glass interface is propagated through the glass coverslip substrate and the associated optics, and then projected onto an imaging camera with finite pixel size to generate a calculated PSF of the AuNR luminescence. Unfortunately, this model does not have a closed form expression and must be evaluated numerically. More details on this model can be found in section 4.2.
Figure 4.2  Diagram depicting the in-plane orientation ($\phi$) possibilities between 0º-360º for the long axis of the gold nanorod. The out-of-plane angle ($\theta$) is also depicted and can be between 0º-90º, with 90º corresponding to a rod that is parallel to the sample plane.

In this chapter we compare the results of mapping the location of ligands on the surface of AuNRs when the AuNR luminescence is fit with either the 3-dipole model or a 2-D Gaussian. The dsDNA labeled with a TAMRA fluorescent reporter molecule remains the ligand in these experiments, and we will continue to use the triplet-state mediated technique to photoswitch the TAMRA molecules for our super-resolution experiments.1,2 Although the 3-dipole model is more computationally expensive than the 2-D Gaussian, we expect it to yield more accurate results for localizing ligands on the surface of AuNRs.

4.2 Data Processing Details

The data consists of *.tiff stacks with 3,649 frames per movie and is processed using in-house written MATLAB code. The data analysis process is described in detail in previous work and will only be briefly summarized here.2 The first frame of each movie is discarded due to timing errors of the CCD camera. To analyze the data, first we differentiate between off frames (AuNR luminescence only, $I_{\text{AuNR}}$) and on frames (TAMRA emission plus AuNR luminescence, $I_{\text{combined}}$) by subtracting adjacent frames. If signal remains in the subtracted image, then this is initially identified as an “on” event; otherwise, the event is initially labeled as an “off” event. Next, the emission intensities from all “off” events, which should be associated with AuNR
luminescence only, are placed into a histogram; the intensity is expected to be normally distributed around some mean value if only AuNR luminescence is present. However, we find significant high intensity outliers in this step, indicating that several “on” frames have been mis-assigned. To reject these frames, we only keep “off” frames that are a single standard deviation above the mean luminescence intensity. Even after this rejection step, we find the luminescence intensity data is skewed from a normal distribution towards higher intensity values. We histogram these data and fit them to a Gumbel distribution, then further reject any “off” frames that are one standard deviation above the mean of the fit to this distribution. This strategy ensures that we are only assigning “off” frames associated with AuNR luminescence and are not biased by images in which a weak TAMRA emitter is also present. Next, we identify “on” events as those that are five standard deviations above the mean AuNR luminescence. Although this strategy will reject weak TAMRA emitters, those events have sufficiently low signal-to-noise that they will be not be well-fit to a 2-D Gaussian for the super-localization analysis and would be rejected later in the data processing.

The “off” frames are then fit with either the 3-dipole model or 2-D Gaussian to calculate the average AuNR intensity and position and allowing us to reconstruct the contribution of the AuNR to each image; this will be referred to as the average AuNR contribution (I_{AuNR,fit,avg}). The average AuNR contribution is then subtracted from each on frame, leaving only TAMRA emission (I_{TAMRA} = I_{combined} - I_{AuNR,fit,avg}). The remaining TAMRA emission is fit with a 2-D Gaussian using a bounded nonlinear least squares method to obtain its centroid position with respect to the AuNR surface. Only fits with R^2 values above 0.8 are kept. The series of movies that are collected for each functionalized AuNR are processed separately. We use the average AuNR luminescence position as a reference point to compile all the TAMRA centroid positions that were localized for each movie in order to produce the final scatter plot.

To account for mechanical drift over the course of the experiments, we use fluorescent sky blue beads as alignment markers. Regions must have at least two alignment markers to be analyzed. The emission from each sky blue bead within the region of interest is fit to equation 1,
and a drift correction is calculated for each frame by subtracting the centroid position of each bead at frame N from its centroid position at frame 2 (recall: frame 1 is discarded due to timing issues). The individual correction trajectories are compared by eye to ensure that individual beads are following identical drift trajectories; otherwise, the region of interest is rejected. The drift tends to move in a single general direction within a single movie, although the direction of the drift will vary from experiment-to-experiment. The corrections for each bead are then averaged to yield a correction trajectory over all frames of the movie, which is then used to correct for drift in the calculated centroids for the AuNR and TAMRA emission.

3-dipole model

As described above, the AuNR luminescence is described as the sum of three mutually-orthogonal dipoles at an air-glass interface. The primary dipole is oriented along the long axis of the AuNR with in-plane orientation angle, $\phi$, and the out-of plane angle, $\theta$, (Figure 4.2) and the other two dipoles are defined to be mutually orthogonal to the primary dipole. To model the dipole emission, we have ten variables that are fit: the defocus of the microscope, the wavelength of emission ($\lambda$), $\phi$, $\theta$, the peak luminescence intensity, the background, the centroid position of the AuNR emission ($x_{NR}$, $y_{NR}$), and the contribution of each dipole component to the total emission intensity.\textsuperscript{15} The emission wavelength, $\lambda$, has the same value for all three dipole components, based upon previous work which showed that allowing each dipole to have a unique value of $\lambda$ (e.g. a fit with 12 variables) yielded non-physical results. In addition to the variables that are fit during our data processing, we must also include experimental parameters such as the numerical aperture of the microscope objective; the magnification of the microscope; the refractive indices of the glass coverslip, microscope immersion oil, and imaging medium; and the distance of the AuNR from the surface (set at zero). Initial guesses are put into our fitting code based upon visual inspection, and then a bounded nonlinear least squares minimization is used to fit each image frame. Outputs from the fit to a single frame are then used as initial guesses for the next frame to reduce computation time. In previous work, we
performed this analysis on 59 AuNRs and showed that this approach yields output parameters (ϕ, θ, λ) that agree extremely well with their experimentally-measured values.\textsuperscript{15} The results from previous data also showed that, on average, it takes ~1 sec/frame to fit AuNR luminescence to a 2D Gaussian and 18 min/frame when using the 3-dipole model, indicating the computational expense of using this more physically rigorous model with a Dell desktop computer.\textsuperscript{15}

4.3 Modeling AuNR Luminescence

Figure 4.3A shows the optical image of AuNR luminescence only (an “off” frame). The AuNR emission pattern indicates dipole-like emission of the AuNR, which can be seen in the bright central feature and two oriented side lobes.\textsuperscript{6,15,20,20–24} The side lobes, which are perpendicular to the long axis of the AuNR, provide a visual aid to assess the orientation of the long axis of the AuNR.\textsuperscript{6,20,23,25–27} We included the correlated AFM image of this AuNR, Figure 4.3B, to show that we are imaging a single AuNR and that the orientation of the AuNR in the AFM matches the orientation of the side lobes in the optical image. The 3-dipole fit for this off frame is shown in Figure 4.3C. The fit was able to accurately model the bright central feature as well as the two oriented side lobes. To further assess how accurate the 3-dipole fit is we create fit residuals (image – fit). The residuals in Figure in 4.3D are random and have low intensity relative to the intensity of the original image. This indicates that the 3-dipole model correctly models the original optical image. Figure 4.3E shows the 2-D Gaussian fit to the AuNR optical data, which is only able to model the central feature. The 2-D Gaussian fit residuals, Figure 4.3F, are non-random with high relative intensities in comparison to the 3-dipole fit, indicating that the 2-D Gaussian is a poor model for this emitter.
Figure 4.3  (A) Optical image of AuNR luminescence. (B) Correlated AFM image of the AuNR show in (A). (C) 3-dipole fit to the AuNR optical image. (D) 3-dipole fit residuals (image-fit). (E) 2-D Gaussian fit to the AuNR optical image. (F) 2-D Gaussian fit residuals (image-fit). Images A, C, D, E, and F share a common 500 nm scale bar. Image B has 50 nm scale bar.

4.4 EFFECT OF AuNR LUMINESCENCE MODELING ON TAMRA EMISSION

Figure 4.4 depicts how the choice of AuNR luminescence model can affect the TAMRA emission contribution ($I_{TAMRA}$) that is calculated after subtracting the average AuNR contribution. Figure 4.4A shows a representative integrated intensity vs. frame (time) trace of the functionalized AuNR from in Figure 4.4. The red dots indicate frames that our algorithm identifies as “on” ($I_{combined} = I_{TAMRA} + I_{AuNR}$) and the green dots indicate “off” frames (AuNR luminescence only). We include a zoomed-in portion of the time trace, inset in Figure 4.4A, to provide a clear view of the steady AuNR luminescence background and the TAMRA emission intensity bursts above that background level (note that some frames are not marked as either “on” [red dot] or “off” [green dot], indicating that the signal did not allow us to make a clear
assignment; these frames are not included in the analysis). The optical image shown in the inset corresponds to frame 2,487 of this movie and is assigned as an “on” frame. We subtracted the average AuNR contribution (I_{AUNR,fit,avg}) from the image in Figure 4.4A, using either the 3-dipole or 2-D Gaussian model, to see how the remaining TAMRA contribution (I_{TAMRA}) was impacted by the choice of model. Figure 4.4B is the average AuNR luminescence contribution, as fit with the 3-dipole model. After subtracting away the 3-dipole modeled average AuNR contribution from the original image from Figure 4.4A, we are left with the contribution from TAMRA emission (I_{TAMRA} = I_{combined} – I_{AuNR,fit,avg}, Figure 4.4C); we will refer to this as the subtracted image. The subtracted image is then fit with a 2-D Gaussian, Figure 4.4D, in order to extract the centroid position of the reporter molecule. The residuals in Figure 4.4E represent the subtracted image minus its 2-D Gaussian fit. These fit residuals are fairly random and have a low relative intensity, indicating that a 2-D Gaussian was able to model the remaining TAMRA emission after the 3-dipole modeled AuNR contribution was subtracted from the original image. The TAMRA emission fit has an R^2 value of 0.85, which passes our R^2 threshold of 0.8, so its centroid position would be included in the final reconstructed image for this functionalized AuNR.
Figure 4.4  (A) Representative integrated intensity vs frame number (time) trace for a TAMRA-labeled AuNR. The red dots signify frames identified as TAMRA emission events (“on” events) and the green dots signify AuNR luminescence only (“off” events). The inset shows a zoomed-in portion of the trace, as well as the optical image for an “on” event at frame 2487. (B) AuNR contribution, calculated with the 3-dipole model. (C) TAMRA emission contribution to this on event ($I_{\text{TAMRA}} = I_{\text{combined}} - I_{\text{AuNR,fit,avg}}$), where the AuNR luminescence is fit with the 3-dipole model and subtracted from the combined image. (D) 2-D Gaussian fit and (E) fit residuals for the subtracted image from (C). (F) AuNR contribution, calculated with the 2-D Gaussian model. (G) TAMRA emission contribution to this on event ($I_{\text{TAMRA}} = I_{\text{combined}} - I_{\text{AuNR,fit,avg}}$), where the AuNR luminescence is fit with the 2-D Gaussian model and subtracted from the combined image. (H) 2-D Gaussian fit and (I) fit residuals for the subtracted image from (G). Images B-I and the optical image in A share a common 500 nm scale bar.
For comparison, we also fit the average AuNR luminescence contribution with a 2-D Gaussian, Figure 4.4F, which does not accurately capture the emission features of the AuNR, as shown in Figure 4.3. This can be seen in the subtracted image, Figure 4.4G, where the two side lobes from the AuNR luminescence emission pattern remain apparent. When the subtracted image (Figure 4.4G), which should only contain the contribution from TAMRA emission, is fit with a 2-D Gaussian (Figure 4.4H), the resulting residuals (Figure 4.4I) have large, nonrandom features with higher relative intensity values than the residuals in Figure 4.4E, indicating a poor quality of fit. This fit, which has an $R^2$ value of 0.7, would not pass our quality-of-fit threshold and thus this TAMRA centroid data point would be excluded from the final reconstructed image.

4.5 Effect of AuNR Luminescence Modeling on Final Reconstructed Images

The process described in Figure 4.4 for a single frame of one of our movies is repeated for all images acquired for this AuNR example. The calculated TAMRA centroid positions are shown as scatter plots in Figures 4.5A and 4.5B. A total of 43,788 frames were taken of this functionalized AuNR and of those frames, 904 were found to be significant “on” events based on the fluorescence intensity. Figure 4.5A shows the calculated TAMRA centroid positions of 837 TAMRA emission events after the 3-dipole modeled AuNR contribution has been subtracted off. In this case, more than 90% of the identified “on” events were successfully fit. On the other hand, Figure 4.5B shows that only 252 (28%) TAMRA emission events are successfully fit when the AuNR contribution is modeled as a 2D Gaussian, indicating that more than 70% of the TAMRA fluorescence data is excluded. To construct these scatter plots we used a quality-of-fit threshold for the 2D Gaussian fits of TAMRA emission events and only accepted fits with a $R^2$ value greater than 0.8. Figure 4.6 shows the reconstructed images created when no quality-of-fit threshold is used. It should be noted that a TAMRA emission centroid position, from a low signal-to-noise fluorescent event, located 100s of nanometers away from the bulk of the centroid positions was excluded from both scatter plots and spatial frequency maps as an obvious outlier in Figure 4.6. TAMRA emission events closer to the functionalized AuNR with low signal-to-
noise lead to poor 2D Gaussian fits, therefore, hindering the precision of the calculated centroid positions, and providing more inaccuracies on the location of dsDNA bound to the AuNR surface.

Figure 4.5 (A) Scatter plot showing centroid positions of TAMRA emission (blue dots) and the average AuNR luminescence contribution (red x) fit with the 3-dipole model. (B) Scatter plot showing centroid positions of TAMRA emission (blue dots) and the average AuNR luminescence contribution (red x) fit with a 2-D Gaussian model. In (A) and (B), N is the number of TAMRA centroid positions that pass our goodness-of-fit criterion and ϕ (orange line) is the in-plane orientation of the AuNR calculated from the 3-dipole model. (C) and (D) Spatial frequency histograms for the data shown in (A) and (B), respectively. The white x is the average (C) 3-dipole-modeled and (D) 2D Gaussian-modeled AuNR position. (E) and (F) Cumulative distribution plots of the centroid positions along the long axis of the AuNR in (A) and (B) respectively. The magenta Xs and black diamonds represent data points on opposite sides of the calculated average luminescence position (white x from C and D). (G) Correlated AFM image of the underlying AuNR, the black line represents the same calculated ϕ as shown in (A) and (B). The out-of plane (θ) tilt angle for each AuNR is also included. (A), (B), (C), and (D) share a common 50 nm scale bar and the scale bar in (G) is 50 nm.
Figure 4.6  Scatter plot and spatial frequency histogram map of TAMRA emission events without implementing a quality-of-fit threshold. The AuNR luminescence contribution was approximated using the 3-dipole model (left) and the 2D Gaussian (right).

Importantly, both scatter plots show a rod-like shape with the correct orientation, as determined both by the correlated AFM image (shown again in Figure 4.5G) and the calculated in-plane dipole angle that is generated by the 3-dipole fit ($\phi = 48^\circ$, orange dashed line in Figures 4.5A and 4.5B and black dashed line in Figure 4.5G). However, due to the ability of the 3-dipole model to better approximate the AuNR dipolar emission, we have many more centroid data points in the reconstructed images and we therefore gain a more complete map of the apparent locations of the dsDNA on the AuNR surface. Another way to visualize the apparent dsDNA locations is to create spatial frequency histograms, Figures 4.5C and 4.5D. The data from the scatter plots are binned in 4.4 nm x 4.4 nm bins (1/10 of an imaging pixel) and the centroid positions that fall within a particular bin are counted. The color map then represents the number of centroid positions in a particular bin and makes it easier to visually differentiate between areas that have more centroid positions localized than others. Again, due to the larger number of
centroid positions we were able to localize using the 3-dipole model compared to the 2-D Gaussian model, we find a more robust image of the underlying AuNR in Figure 4.5C than 4.5D. The white x’s in the spatial frequency maps (and the red x in the scatter plots) represent the average AuNR luminescence centroid position as determined by the different models. We find that the AuNR luminescence position is located in two distinctly different places between the two fitting approaches. We assume that the average AuNR luminescence position should represent the center of the AuNR and would therefore be in the center of the TAMRA centroid positions if we are achieving uniform dsDNA coverage. While this is the case for the data fit with the 3-dipole model (Figure 4.5C), the luminescence centroid is biased towards the upper left of the rod for the data calculated with the 2-D Gaussian (Figure 4.5D). We can understand this difference by analyzing the out-of-plane tilt angle, θ, calculated using the 3-dipole model, which tells us that the AuNR is titled slightly out-plane at 87º. Previous work has shown that the accuracy of using a 2-D Gaussian to calculate the correct centroid position from a dipole emitter will decrease if the emission being fit corresponds to an emitter that is tilted out-of-plane.15,29,30 In this case, the out-of-plane tilt of the rod leads to an incorrect location of the AuNR luminescence centroid, which is obvious from the data shown in Figure 4.5D.

To compare the calculated size of the reconstructed images to the actual size of the AuNR, we calculated cumulative distribution functions (CDF), as shown in Figures 4.5E and 4.5F. Briefly, the data in the centroid position scatter plots (Figures 4.5, A and B) are rotated such that the long axis of the AuNR lies on the x-axis. Figure 4.7 is included to show details on how these CDF plots are constructed. The long axis of the scatter plot shown in Figure 4.7A (which is the same scatter plot shown in Figure 4.5A), was rotated around zero, the average luminescence position (modeled using the 3-dipole code), onto the x-axis, Figure 4.7B. The rotation angle was dictated by the in-plane angle, ϕ, calculated using the 3-dipole code. To construct these plots we start at the marked zero position (shown in the red line for x and black line for y).
Figure 4.7  (A) Scatter plot from 4.5A showing TAMRA centroid positions. (B) Scatter plot (showing same data as (A)) after the long axis was rotated by $\phi$ around the 3-dipole modeled AuNR luminescence position, to yield the long axis of the nanorod oriented along the x-axis. The red dotted line corresponds to the x AuNR luminescence centroid position and the black dotted line corresponds to the y AuNR luminescence centroid position (arbitrarily set at (0,0)). (C) and (D) Cumulative distribution function plots constructed for centroid positions along the long and short axis of the AuNR. The magenta Xs represent positive distances and black diamonds represent the absolute value of negative distances.

Figure 4.8  The cumulative distribution function plot showing centroid positions along the short axis for the data shown in 4.4 B.
For the CDF plot showing the centroid positions along the long axis of the AuNR, Figure 4.7C, we count the centroid points with an x value at zero only and divide that number by the total amount of centroid points. That fraction is plotted in the CDF plot at zero. Next, the centroid points with an x value between 0 and 4 nm are counted and that number is divided by the total amount of centroid points. That fraction is plotted in the CDF plot at 4 nm. The intervals increase by 4 until all the x values in the centroid scatter plot are accounted for. We chose the interval of 4 nm because it corresponds to the same bin size we use to create the spatial frequency histograms shown in the main text. The CDF plots for points on either side of zero were created separately, and the absolute value of the negative distances were used in order to display the information on the same plot. Points are plotted as either black diamonds or magenta Xs to show points that fall on either side of the AuNR center and highlight any asymmetry in the reconstructed AuNR relative to the calculated luminescence centroid. The same process is used to create the CDF plots showing the centroid positions along the short axis of the AuNR, Figure 4.7D, but it corresponds to the y value for each of the centroid positions.

We expect the CDF to plateau at its maximum value of 1 at a distance from center that corresponds to half the nanorod length (∼80/2 = 40 nm) plus the length of the DNA spacer (∼10 nm) for a total distance of 50 nm. From the CDF plot in Figure 4.5E (identical to the plot in Figure 4.7C), in which the 3-dipole model was used to fit the AuNR luminescence, the plateau happens ∼30 nm from the AuNR center, well below the expected distance of 50 nm. Thus, even when using this improved model for the AuNR luminescence, we still find reconstructed images with dimensions smaller than the expected size of the nanorod (the corresponding CDF plot for the nanorod width is shown in Figure 4.7D), consistent with our previous work. The data on either side of center (black vs magenta points) are symmetric, due to the fact that the AuNR luminescence average centroid position (as determined via the 3-dipole model) is centered with respect to the TAMRA centroid positions, as seen in Figure 4.5C.

When the 2-D Gaussian model is used to model the AuNR luminescence, we see the data in the CDF plot (Figure 4.5F) are not symmetric due to the AuNR luminescence centroid average
position not being centered with respect to the TAMRA centroid position. For Figure 4.5F, the black data plateaus at ~15 nm and the magenta line plateaus at ~30 nm yielding a total reconstructed AuNR length of ~45 nm (reconstructed width is ~15 nm, CDF plot shown Figure 4.8). This is even smaller than the reconstructed dimensions calculated using the 3-dipole model. The dimensions of the AuNR from the AFM image, Figure 4.5G, was found to be ~80 nm x 27 nm after accounting for tip effects that cause size over-exaggeration. When the ~9.5 nm dsDNA linker length is taken into account, the expected dimension for this functionalized AuNR is ~99 nm x 46 nm. We discussed this size mismatch in previous work and hypothesized that a better AuNR model may help fix this issue; however, these data indicate that even with this improved model, we still reconstruct images well below the actual size of the nanorod. The size mismatch is also shown in Figure 4.9 where the reconstructed images in Figure 4.5C and D, and the corresponding AFM image, Figure 4.5G, are scaled to be on the same size scale and are qualitatively overlaid upon each other. The reconstructed images are clearly smaller than the AFM image, even when we take into account the size over-exaggeration caused by tip affects from the AFM (the corrected size is marked with the gray line). The width of the reconstructed functionalized AuNR image, when using the 3-dipole, provides a closer agreement to the expected dimension than when using the 2D Gaussian, however, the length is greatly undersized in both cases (a recurring trend throughout this chapter).

Figure 4.9 Reconstructed images from 4.5C (left) and 4.5D (right) and the AFM image from 4.5G were scaled to an equal size and overlaid to show the size mismatch between expected and reconstructed dimensions. The gray outline represents the expected functionalized AuNR dimensions, after taking into account AFM tip effects.
Figure 4.10  Left column (A, D, G, and J): Spatial frequency histograms showing TAMRA emission for four different AuNRs. The AuNR luminescence was fit with the 3-dipole model and the average location is shown as a white x. Middle column (B, E, H, and K): Spatial frequency histograms for the same four AuNRs, with the AuNR luminescence fit with a 2-D Gaussian. The white x represents the average AuNR luminescence centroid calculated with the 2-D Gaussian model. Right column (C, F, I, and L): Correlated AFM images of the underlying AuNRs. The black line represents the calculated $\phi$ from the 3-dipole model. The out-of-plane ($\theta$) tilt angle for each AuNR is also included. Each row corresponds to the same TAMRA-labeled AuNR. N equals the number of TAMRA centroid positions that were localized. All the spatial frequency histograms share a common 50 nm scale bar, and the AFM images have a 50 nm scale bar.
Figure 4.10 shows a montage of different AuNRs illustrating the effects of fitting the AuNR contribution with the 3-dipole model (left column) or the 2-D Gaussian model (middle column) on the calculated spatial frequency histograms. The left column, Figures 4.10A, 4.10D, 4.10G, and 4.10J, shows spatial frequency histogram maps that have been constructed from “on” events in which the 3-dipole modeled AuNR luminescence contribution has been subtracted away, leaving only TAMRA emission. The second column, Figures 4.10B, 4.10E, 4.10H, and 4.10K, shows spatial frequency maps that have been constructed using 2-D Gaussian modeled AuNR luminescence to subtract the AuNR contribution from the TAMRA emission. The histogram maps in each row correspond to the same functionalized AuNR. The number of TAMRA emission centroid positions that were used to create the histogram maps is included on each map. More centroid points pass our quality-of-fit screening when the 3-dipole model was used than the 2-D Gaussian model, consistent with the trend from Figure 4.4. The correlated AFM images for each AuNR are in the right column, Figures 4.10C, 4.10F, 4.10I, and 4.10L. The white x in each spatial frequency histogram marks the average AuNR position calculated with the different models. As with Figure 4.4, we see a larger offset between the luminescence centroids calculated with the two different models as the out-of-plane tilt (θ) increases.

Figure 4.10 shows that the spatial frequency histograms show diversity in the locations of the TAMRA emission, suggesting local heterogeneity in the binding of the dsDNA. For example, the top row of Figure 4.10 (A-B) shows more activity on the ends of the nanorod, with a dearth of activity near the center; this behavior is also apparent in the second row. In the second row, there are more centroid localization events in the upper right portion of the nanorod than the lower left, suggesting either more TAMRA-labeled dsDNA at this site or more active TAMRA emitters. This trend is also apparent in the other examples, particularly the data shown in the third row, where the upper portion of the AuNR shows much more TAMRA activity than the lower portion. Similar behavior has been previously observed by us. What is of particular note here is that this apparent asymmetry is present regardless of the model used to fit the AuNR data, and that there is excellent qualitative agreement between TAMRA activity observed in the
spatial intensity maps using either the 3-dipole fit model (Figure 4.10, left column) or the 2-D Gaussian model (Figure 4.10, center column). This suggests that the asymmetry is not an artifact of the model, but is instead a real experimental observation associated with local heterogeneity in the binding/activity of the TAMRA-labeled dsDNA.
Figure 4.11 Cumulative distribution function plots, for data along the long (titled length) and short (titled width) AuNR axis, created using the data from each reconstructed image previously discussed. The luminescence fit to the 3-dipole model (left) or a 2-D Gaussian model (right). The Xs and diamonds represent data points on opposite sides of the calculated average luminescence position. Color legend: magenta corresponds to the data used to reconstructed image in Figure 4.5 A-D, blue corresponds to the data used to create the reconstructed images in Figure 4.10 A-B, green corresponds to the data used to create the reconstructed image in Figure 4.10 D-E, orange corresponds to the data used to create the reconstructed images in Figure 4.10 G-H, and black corresponds the data used to create the reconstructed images in Figure 4.10 J-K.
The CDF plots, showing data along the long and short axis of the AuNRs, for each of the reconstructed images discussed in this chapter are displayed in Figure 4.11 for comparison. We chose to use CDF plots because they allow for easier data analysis of reconstructed image dimensions, and we can combine all the CDF plots from a data set, Figure 4.12, for easy viewing and comparison of multiple functionalized AuNRs on a single plot. Once again, we find that the sizes of the reconstructed AuNRs are well below the expected dimensions, regardless of the model used. Figures 4.12A and 4.12B are the length and width CDF plots that were created from the TAMRA centroid data in which the 3-dipole modeled AuNR luminescence was subtracted off, while Figures 4.12C and 4.12D show the length and width CDF plots when the AuNR was modeled as a 2-D Gaussian. The overall trend in the length CDF plots, Figures 4.12A and 4.12C, is that the data points plateau sooner than the expected distance from center. The average size of the AuNRs used in this experiment is $89 \pm 8 \text{ nm} \times 30 \pm 2 \text{ nm}$ with an aspect ratio of $\sim 3$. This means that the reconstructed length should be $\sim 107 \text{ nm}$ when dsDNA linker length is taken into account. Ideally, if we were reconstructing the proper length, we would expect the plateau to happen around $\sim 54 \text{ nm}$ for data points on both sides of zero if the lines were symmetric, yet we observe that 100% of the points are accounted for at $\sim 30 \text{ nm}$ from center for all examples shown, regardless of model. This same trend is also evident in the width CDF plots, Figures 4.12B and 4.12D. The reconstructed images should be $\sim 49 \text{ nm}$ wide when the dsDNA linker taken in to account. This means the data points should not plateau until $\sim 25 \text{ nm}$ for data on both sides of zero, assuming the lines are symmetric. Monte Carlo simulations have been done to model our experimental results and we see similar CDF plots when simulating these exact experimental conditions, this will be discussed in further detail in a future publication. In the CDF plots using either the 3-dipole (Figure 4.12B) or 2-D Gaussian (Figure 4.12D) models, the widths are still below the expected value, although we find that the 3-dipole model yields larger overall widths than the 2-D Gaussian, suggesting at least some improvement in the reconstructed size when using this fit.
**Figure 4.12** Cumulative distribution function plots depicting centroid positions along the long (A) and short (B) axis of the AuNR, luminescence fit to the 3-dipole model. Cumulative distribution function plots depicting centroid positions along the long (C) and short (D) axis of the AuNR, luminescence fit to a 2-D Gaussian model. Within a given cumulative distribution plot, each color corresponds to a different AuNR. Among all four plots, each color corresponds to the same functionalized AuNR example. The Xs and diamonds represent data points on opposite sides of the calculated average luminescence position. Color legend: magenta corresponds to the data used to reconstructed image in Figure 4.5 A-D, blue corresponds to the data used to create the reconstructed images in Figure 4.10 A-B, green corresponds to the data used to create the reconstructed image in Figure 4.10 D-E, orange corresponds to the data used to create the reconstructed images in Figure 4.10 G-H, and black corresponds to the data used to create the reconstructed images in Figure 4.10 J-K.

### 4.6 Conclusions

In this chapter we compared the effects of different AuNR luminescence fit models on the reconstructed images of fluorescently-labeled dsDNA bound to the surface of AuNRs. Table 4.1 tabulates the results as a concise summary. By using the 3-dipole model we were able to pass more TAMRA emission events through our fitting threshold to get a more robust map of the
apparent locations of bound dsDNA in the reconstructed images, and the model provides useful output parameters such as $\theta$ and $\phi$ for the underlying AuNR. Unfortunately, subtracting off a better modeled AuNR contribution did not fix the size mismatch issues between the expected AuNR dimensions and the dimensions of the functionalized AuNR in the super-resolution reconstructed images. However, we do find that the reconstructed images yield aspect ratios that are consistent with the aspect ratio of the labeled AuNRs. We believe that other factors are contributing to this size-mismatch issue, such as altered TAMRA photophysics caused by plasmonic interactions with the AuNR that can change the triplet state lifetime of TAMRA and/or induce TAMRA-AuNR coupling\textsuperscript{10} or the formation of image dipoles within the AuNR that can perturb the output point spread function\textsuperscript{31}. Both situations will impact the reconstructed images of the labeled AuNRs by causing smaller than expected images; however, more experiments are needed in order to fully understand the magnitude of their impacts.
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<th>$\theta$ (°)</th>
<th>$\lambda_{\text{scattering, max}}$ (nm)</th>
<th>Dimensions (l x w) from AFM (nm)</th>
<th>Expected Dimensions accounting for DNA linker length (nm)</th>
<th>3-dipole Dimensions (l x w) from CDF plots (nm)</th>
<th>2D Gaussian Dimensions (l x w) from CDF plots (nm)</th>
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<td>~91 x ~29</td>
<td>~111 x ~49</td>
<td>~60 x ~35</td>
<td>~60 x ~30</td>
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<td>~60 x 30</td>
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</table>

Table 4.1 Tabulated results for the functionalized AuNRs study in this chapter.

Despite the smaller-than-expected reconstructed images, we were able to accurately model AuNR luminescence and TAMRA emission within a diffraction-limited spot to map the position of the AuNR and gain insight into the apparent heterogeneity of dsDNA on the AuNR surface. With both models, we observed similar asymmetry in terms of apparent dsDNA binding locations in the spatial frequency histograms, suggesting that the observed binding heterogeneity is real and not a fit artifact. Finally, we showed that the luminescence signal can serve as a benchmark to combine multiple data sets together; therefore, data collection is only limited to how long TAMRA molecules continue to undergo photoswitching. This increases the likelihood of probing all the dsDNA that are attached to the AuNR surface. By fitting the luminescence to
a 3-dipole model, we extract multiple parameters associated with the AuNR orientation, which will ultimately negate the need for correlated AFM imaging in order to confirm that our super-resolution image correctly maps the underlying shape of the AuNR support.

4.7 Acknowledgements

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4.8 References


In previous research from our group, we have mapped the apparent location of fluorescently-labeled double-stranded DNA (dsDNA) on the surface of gold nanorods (AuNRs).¹ We used a triplet-state mediated technique to photoswitch the fluorescent tags between an emissive ‘on’ state and a non-emissive ‘off’ state.²,³ The emission from a fluorophore in the ‘on’ state was fit to a two-dimensional (2D) Gaussian to extract the approximate location of the emitter. This location indirectly provided the approximate position of where a dsDNA was bound to the surface of the AuNR. This procedure was done repeatedly to attempt to probe all the fluorescent labels on the AuNR surface, and we have observed the heterogeneous nature in which ligands bind to AuNRs from solution. We based our conclusion of dsDNA binding heterogeneity on the non-uniform distribution of fluorescence emission centroid positions with respect to the AuNR surface. We also used the fluorophore centroid positions to build reconstructed images of individual labeled-AuNR substrates, which we used to report on the shape and orientation of the AuNRs. Unfortunately, in all of the reconstructed images thus far we have found that the size of the optically-reconstructed AuNRs is smaller than expected based on correlated atomic force microscopy or electron microscopy images.

In Chapter 4, we attempted to address this issue by using a more rigorous dipolar model to approximate the AuNR luminescence contribution.⁴,⁵ Subtracting a well-modeled luminescence contribution from the emission of a molecule in the ‘on’ state is integral to our data processing. Failure to do so results in the fluorophore centroid position being skewed towards the AuNR position, effectively shrinking the size of the reconstructed image. Modeling the luminescence as three mutually-orthogonal dipole modes (details about this model can be found in Chapter 4) provided several benefits, such as better AuNR position approximations, more robust reconstructed images with more fluorophore centroid positions, and useful output
parameters about the underlying AuNR 3-dimensional orientation. Unfortunately, the size mismatch issue was not resolved using this more rigorous model, leading us to consider other hypotheses. Research conducted in another lab has indicated that the point spread function (PSF) of the fluorophore can be distorted because of its proximity to a metal surface and these distortions will lead to inaccuracies when fitting a PSF image taken near a metallic surface.\textsuperscript{6,7} We have ruled this out as an explanation because we do not visually observe the large distortions in the raw PSF data collected in our experiments, unlike the published reports.\textsuperscript{1,8}

In this Chapter we discuss several experimental attempts to address the size mismatch issue by addressing the photophysics of the fluorophore system. Two strategies involve lowering the probability of multiple fluorophores emitting simultaneously. If multiple emitters are in the ‘on’ state at the same time, we fit a super-position of that emission which yields an intensity-weighted centroid position that does not reflect the true position of each individual emitter. For fluorophores spaced on opposite sides of the AuNR, this super-position effect will move the calculated centroid towards the center of the nanorod, leading to a smaller-than-expected size in the reconstructed nanorod image. For the triplet-state mediated photoswitching strategy that we use in our experiments, we want all but one of the fluorophores shelved in the triplet state at a given time, to promote only a single emission event within a given diffraction-limited spot. Experimentally, we have tried to control this by using high enough laser excitation to promote intersystem crossing (without melting the AuNR) and by working under a nitrogen environment to eliminate triplet-state scavenging oxygen so the molecules will reside longer in the triplet state. In other work, thiols are also used to help promote longer-lived dark states, by promoting formation of metastable dark species, but in our system, introducing thiols interferes with the binding of the thiolated dsDNA to the gold surface.\textsuperscript{9–11} Unfortunately, this leaves us with a limited number of experimental knobs for tweaking. Therefore, we tested several other ways to lower the probability of emitters fluorescing simultaneously, both by changing the number of fluorophores tethered to the AuNR and by changing the identity of the fluorophore from TAMRA to one with a longer expected triplet state lifetime. As an alternate strategy, we also
increased the length of the dsDNA linker to test whether proximity to the Au surface could be impacting the TAMRA photophysics.

5.2 TAMRA-dsDNA Concentration Study

First, we altered the concentration of fluorescently labeled-dsDNA available to bind to the AuNRs in solution. Ideally, having a lower amount of fluorophores available to bind to the AuNR should result in a lower number of fluorophores available to simultaneously emit once tethered to the nanorod surface. The first concentration, which will be referred to as the low concentration, was made by combining 0.72 nM TAMRA-dsDNA and 7.2 µM unlabeled-dsDNA in 100 µL with the AuNRs (100 µL of the overgrown AuNR solution was pelleted via centrifugation and resuspended in the dsDNA solution following the functionalization protocol discussed in Chapter 2). The low concentration level provides only one TAMRA-dsDNA for every 10,000 unlabeled-dsDNA. When using this dilution level, we expect to see very low numbers of binding events occurring on AuNRs, meaning that there should be very few TAMRA emission events to fit. The second concentration, which will be referred to as the mid concentration, was made by combining 7.2 nM TAMRA-dsDNA and 7.2 µM unlabeled-dsDNA in 100 µL with the AuNRs. In this level the ratio of TAMRA-dsDNA to unlabeled-dsDNA is 1:1,000. The mid concentration level was used in previous experiments to create the reconstructed images shown in Chapter 4 and we will provide more examples at this concentration in this section. The last concentration, denoted the high concentration, was made by mixing 3.6 µM of TAMRA-dsDNA and 3.6 µM of unlabeled-dsDNA, creating a 1:1 ratio. This ratio was chosen because, ideally, each dsDNA should have an equal opportunity to bind to the surface of an AuNR so this would be a way to observe competitive cooperative binding. However, in this high concentration regime, we must be mindful that inter-dye fluorescence quenching can occur. The length of the dsDNA in these experiments was ~9.52 nm, putting our TAMRA molecule well out of the quenching regime of the AuNR. This distance will also be referred to as the original dsDNA linker length later in this chapter.
Figure 5.1  Reconstructed images created using the low (1:10,000) concentration of TAMRA-dsDNA. Each spatial frequency histogram map (blue background) has a corresponding spatial intensity map (black background). The spatial frequency maps suggest homogeneous dsDNA binding on AuNRs in solution. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar. Bin sizes in the reconstructed images equals 4.4 x 4.4 nm for A-F and 5.3 x 5.3 nm for G-H.

Figure 5.2  Reconstructed images created using the low (1:10,000) concentration of TAMRA-dsDNA. Each spatial frequency histogram map (blue background) has a corresponding spatial intensity map (black background). The spatial frequency maps suggest that sparse labeled-dsDNA binding events occurred on the AuNR surfaces. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar. Bin sizes in the reconstructed images equals 4.4 x 4.4 nm for A-F and 5.3 x 5.3 nm for G-N.
Figure 5.1 depicts examples of reconstructed images created from imaging the low concentration labeled-AuNRs. The spatial frequency histogram maps, shown with the blue background, were created by counting the amount of TAMRA emission centroid positions located in a particular region of space, or bin (bin sizes equals 4.4 x 4.4 nm for A-F and 5.3 x 5.3 nm for G-H, both correspond to 1/10 of an imaging pixel and is dependent on which EM-CCD camera was used for data collection). The color scale bar next to the spatial frequency maps indicates how many centroid positions are counted in each bin. The spatial intensity maps, shown with the black background, were created by plotting the average intensity of the TAMRA emission events in each bin of the associated spatial frequency maps. The scale bar next to these images represents average fluorescence intensity. We included the spatial intensity maps to show that that we are not observing intensity enhancement across the AuNR surfaces that would indicate surface-enhanced fluorescence and fluorophore-AuNR coupling.

The spatial frequency maps in Figure 5.1 show rod-like reconstructed images. This is unexpected due to the low concentration of TAMRA-dsDNA that was added, which should lead to fewer than one fluorescent label per rod based on calculations of the average DNA footprint on a nanorod surface. Thus, these results suggest cooperative binding, in which it is possible that once a TAMRA-dsDNA binds, it helps facilitate other binding of the same type of dsDNA, leading to a non-stoichiometric number of TAMRA-dsDNA binding events on the AuNR. Nevertheless, the majority of the reconstructed images show sparse spatial frequency maps that do not portray a rod-like shape, as in Figure 5.2. The small number of TAMRA emission localized across the surface of AuNR may indicate few binding events, which is expected with the low concentration of TAMRA-dsDNA that was used. The white ‘x’ in the spatial frequency maps represents the average AuNR luminescence for each example. The luminescence appears to be centralized with respect to the TAMRA emission centroid positions in the examples that show rod-like reconstructed images, Figure 5.1. If we examine the sparse maps (Figure 5.2), we also find the white ‘x’ tends to be somewhere near the middle of the TAMRA centroid data, indicating that the events are due to bound TAMRA-dsDNA rather than non-specific emission.
events. The results of the low concentration TAMRA-dsDNA show that the reconstructed images are still smaller than expected. Also, the reconstructed images in Figures 5.1, G-H, and Figures 5.2, G-N, were constructed using 29,192 more frames of raw data than the images in Figures 5.1, A-F, and Figures 5.2, A-F, but the extra frames did not make a difference in the number of TAMRA emission events that were localized, suggesting that this number is limited by the number of fluorophores bound to the surface.

Figure 5.3  Reconstructed images created using the mid concentration (1:1,000) of TAMRA-dsDNA. Each spatial frequency histogram map (blue background) has a corresponding spatial intensity map (black background). The spatial frequency maps in this figure suggest evenly distributed TAMRA-dsDNA binding across the AuNR surfaces. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.
Figure 5.4  Reconstructed images created using the mid concentration (1:1,000) of TAMRA-dsDNA. The spatial frequency histogram map (blue background) has a corresponding spatial intensity map (black background). The spatial frequency map in this figure suggests preferential binding of TAMRA-dsDNA binding at the ends of AuNRs. The white ‘x’ in the spatial intensity map represents the average AuNR luminescence position. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.

Figure 5.5  Reconstructed images created using the mid concentration (1:1,000) of TAMRA-dsDNA. Each spatial frequency histogram map (blue background) has a corresponding spatial intensity map (black background). The spatial frequency maps in this figure suggest preferential binding of TAMRA-dsDNA to one end of the AuNR over the other. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.
Figure 5.6  Reconstructed image created using the mid concentration (1:1,000) of TAMRA-dsDNA. The spatial frequency histogram map (blue background) has a corresponding spatial intensity map (black background). The spatial frequency map in this figure suggests preferential binding of TAMRA-dsDNA to one end of the AuNR over the other, leaving the other end completely unlabeled based on the luminescence position. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.

Figures 5.3-5.6 show the reconstructed images created using the mid (1:1,000) concentration labeled AuNRs (more examples can also be seen in Chapter 4). The spatial frequency maps in Figure 5.3-5.5 show rod-like reconstructed images that map the shape and orientation of the underlying AuNRs. With this mid concentration we see examples of the binding heterogeneity that occurs when dsDNA binds randomly to AuNRs in solution; this is based on the frequency of TAMRA centroid positions on different areas of the AuNR surface. For example, the spatial frequency maps Figure 5.3 show evenly distributed centroid points in the shape of the AuNR, suggesting that uniform binding occurred across the AuNR surface. Figure 5.4 shows an example where the majority of centroid points were localized at the ends of the AuNRs, suggesting preferential binding of the TAMRA-dsDNA to the AuNRs ends. The spatial frequency maps in Figure 5.5 show more TAMRA emission localization on one end of the AuNR than the other. The reconstructed image in Figure 5.6 was included to show that sometimes we observe incomplete binding across the AuNR surface even with this concentration level, suggesting that TAMRA-dsDNA did not bind all over the AuNR. Most of the localized TAMRA emission events are located below and to the right of the underlying AuNR luminescence centroid position, marked by the white ‘x’, providing evidence that TAMRA-dsDNA bound only to the lower half of this AuNR. As previously observed, these reconstructed
images are smaller-than-expected when taking into account the dsDNA linker and the size of the AuNR.

The number of centroid positions counted in each of the bins in the spatial frequency maps in the mid concentration data set are notably higher than what was observed for the low concentration results in Figures 5.1-5.2 (30-100 vs. 3-10 max events). We assume that each fluorophore gives the same average number of emission events (e.g. the same fluorophore can be ‘on’ multiple times but that number should be concentration-independent), so this higher overall frequency indicates a higher number of TAMRA-dsDNA bound to the AuNR surface, as expected for this higher labeling concentration. We see that this number is roughly an order of magnitude higher than that observed for the low concentration, supporting this hypothesis.

Figure 5.7  Reconstructed images created using the high concentration (1:1) of TAMRA-dsDNA. The spatial frequency histogram map (blue background) has a corresponding spatial intensity map (black background). The spatial frequency map suggests evenly distributed TAMRA-dsDNA binding events across the AuNR surface. The white ‘x’ in the spatial intensity map represents the average AuNR luminescence position. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.
Figure 5.8 Reconstructed images created using the high concentration (1:1) of TAMRA-dsDNA. Each spatial frequency histogram map (blue background) has a corresponding spatial intensity map (black background). The spatial frequency maps suggest more TAMRA-dsDNA binding events on one side of the AuNR surface. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 5.3 x 5.3 nm.

Figure 5.9 Reconstructed images created using the high concentration (1:1) of TAMRA-dsDNA. Each spatial frequency histogram map (blue background) has a corresponding spatial intensity map (black background). The spatial frequency map shows spare TAMRA emission centroid positions. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm for A-B and 5.3 x 5.3 nm for C-J.
Figures 5.7-5.9 shows examples of reconstructed images from the high concentration TAMRA-labeled AuNRs. For the examples we have at this concentration level, we have observed only one example that suggests even TAMRA-dsDNA binding across the AuNR surface, Figure 5.7. The spatial frequency maps in Figure 5.8 suggest more binding of TAMRA-dsDNA on one end than the other. The other examples in Figure 5.9 suggest the TAMRA-dsDNA did not bind all over the AuNR surface, which is based on the location of the AuNR luminescence centroid position, marked by the white ‘x’. We did not expect to observe so many examples that show this incomplete TAMRA-dsDNA binding with the high concentration of TAMRA. At first, we attributed this to competitive cooperative binding that favors the unlabeled-dsDNA, however, the low frequency of TAMRA events being localized in the spatial frequency maps was a worrying point. Based on the trend we observed between the low and mid concentration data sets, we expected a several orders of magnitude increase in the frequency of TAMRA emission centroid points counted in the spatial frequency maps when comparing the mid and high concentration data sets (1:1,000 vs 1:1). On the contrary, the frequency counts were lower in the high concentration regime, most likely due to the fact that the high number of TAMRA-dsDNA packed closely on the AuNR surface is causing high occurrences of the nearby TAMRA molecules to quench themselves. Therefore, the example in Figure 5.7 likely represents a rare occasion when the 1:1 ratio of labeled and unlabeled dsDNA in solution is preserved on the AuNR surface. The examples in Figure 5.8 likely suggests that competitive cooperative binding, favoring the TAMRA-dsDNA, occurred when the dsDNA were binding to the AuNRs in solution. Figure 5.8A has a higher frequency count than Figure 5.8C, which could mean that the AuNR in Figure 5.8C had more TAMRA-dsDNA packed closely on the one end, resulting in a lot of quenching. This could also explain spatial frequency map in 5.9A where half of the AuNR appears to not have had any bound TAMRA-dsDNA. Also, in Figure 5.9, the appearance of incomplete binding in the spatial frequency maps is likely caused by a combination of cooperative binding that favors the TAMRA-dsDNA and the subsequent quenching that occurs from having too many TAMRA molecules close together.
Finally, we compiled all the examples for each of the TAMRA-dsDNA concentrations into separate cumulative distribution function (CDF) plots, Figure 5.10. The CDF plots are a convenient way to view and compare the reconstructed image data for all three conditions. The CDF plots are also a good way to visualize the heterogeneity of the TAMRA centroid positions localized across the surface of the AuNRs. The CDF plots are ordered by TAMRA-dsDNA:unlabeled-dsDNA ratio, high to low. The CDF plots for the length and width dimensions of the reconstructed nanorod images are shown. In the length CDF plots, the Xs represent the centroid points localized to the right side of the calculated luminescence center and the diamonds represent the centroid points on the left side of the center after the data is rotated by the long axis onto the x-axis. In the width CDF plots, Xs represent the centroid positions with y values above the AuNR luminescence center and diamonds represent y values below the center (Chapter 4 includes more details on how the CDF plots are created). The size of the reconstructed image should be ~108 nm x ~50nm, taking into account the average AuNR size and the length of the dsDNA linker. In the CDF plots for centroids along the length of the AuNR we would expect 100% of the points to be accounted for ~ 54 nm from both sides of the luminescence center; unfortunately all three length CDF plots show that 100% of the data points are accounted for well below this value. In the CDF plots along the short axis of the AuNR, 100% of the points should be accounted for ~25 nm from the center on both sides; however the width dimension is also smaller than expected. In the CDF plots the range and heterogeneity of the centroid positions along the long and short axis of the AuNRs are observed by looking at the different distances from zero where 100% of the centroid points are reached as well as the different shapes of the curves. Overall, while the concentration study provided insight about the differences in TAMRA emission centroid position frequency between the different concentrations, the CDF plots and reconstructed images show that the size mismatch issue was not fixed by reducing the probability of simultaneous emitters on the nanorod surface.
Figure 5.10 Cumulative distribution function plots depicting length and width data for all reconstructed images shown for the concentration study. For each concentration (separated by rows): within a given cumulative distribution plot each color corresponds to a different AuNR, and between the length and width plots each color corresponds to the same functionalized AuNR example. For the length CDF plots: Xs represent the values on the right side of the AuNR luminescence center and diamonds represent the values on the left side of center. For the width CDF plots Xs represent the values above the center and diamonds represent the values below.
5.3 RECONSTRUCTED IMAGES OF ATTO 532-LABELED dsDNA ON AuNRs

The second experimental attempt for addressing the size mismatch issue is to change the fluorescent label to a molecule with a longer lived ‘off’ state. In this case, having the fluorophores in an ‘off’ state for longer keeps the probability of populating the ground state low. Therefore, we have a better chance of having only one fluorophore at a time in the ‘on’ state. For all the experiments discussed thus far in this dissertation, we have used TAMRA as the fluorescent reporter molecule. It has been reported in the literature that TAMRA, when adhered to a glass coverslip, has a triplet-state lifetime of only \( \sim 3.1 \mu s \).\(^{15}\) To achieve a longer ‘off’ time, we switched the reporter molecule to Atto 532, which has a reported dark state time of \( \sim 230 \) ms in poly(vinyl-alcohol).\(^2\) The local environment (e.g. nitrogen, ambient air, poly(vinyl-alcohol), etc.) of the molecule can greatly affect its triplet-state lifetime; therefore the lifetimes reported cannot be directly applied to our experiment. However, they can provide a clue to which molecules might be better suited for the triplet-state mediated imaging technique.

The Atto 532 experiments were conducted using the mid-concentration level (1:1,000 unlabeled:labeled dsDNA) and the same dsDNA linker (~10 nm long) as the TAMRA experiments. Figures 5.11–5.14 show a montage of reconstructed images created using the centroid positions from Atto 532 emission. The spatial frequency histogram maps in Figure 5.11 depict examples where localization of Atto532 emission occurred evenly across the surface of AuNR, suggesting an even binding distribution of the labeled-dsDNA. The white ‘x’ in the maps represents the average AuNR luminescence position for each example. The ‘x’ is centrally located with respect to the Atto 532 emission data points, as expected. Optimistically, the spatial frequency map shown in Figure 5.11A shows the expected dimensions for the dsDNA-labeled AuNR (~108 nm x ~49 nm, taking into account the average AuNR dimensions and the length of the dsDNA). Unfortunately, this is the only example (1 out of 19) of the Atto 532 labeled-AuNRs that had a spatial frequency map with the expected AuNR dimensions. In Figures 5.11, C and E, the spatial frequency maps show reconstructed labeled-AuNR images where the width appears to be accurate; however, the length is still shorter than expected. So far we have not been able to
pinpoint a trend in the data to explain this occurrence. For instance, the localized surface plasmon resonance (LSPR) for each of the underlying AuNRs are different and an orientation artifact is ruled out because there are similarly-orientated rods that are smaller than expected in both length and width. The corresponding spatial intensity maps were included next to each spatial frequency map in Figure 5.11 to show, again, that we did not observe local intensity increases that suggest surface-enhanced fluorescence. The intensity differences across the AuNR surfaces in Figure 5.11 vary on average by a factor of ~3. Although we are not seeing high intensity increases to indicate strong coupling, we are still observing a size mismatch in the reconstructed images.
Figure 5.11  Reconstructed images of Atto 532 labeled dsDNA attached to AuNRs. The spatial frequency histogram maps (blue background) depict evenly distributed Atto 532 emission localization across the AuNR surface. The white ‘x’ in the maps represents the average location of the AuNR luminescence. The corresponding spatial intensity map for each AuNR is shown as well. The images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.
Figure 5.12 shows examples of reconstructed images where the localization of Atto 532 emission happens mostly on the ends of the AuNRs, suggesting the labeled-dsDNA bound preferentially to the ends of the AuNRs. Figure 5.13 shows examples that suggest more labeled-dsDNA binding occurring on one side of the AuNR over the other. Finally, Figure 5.14 shows two examples that indicate incomplete labeling on those particular AuNR. Again, this could be a random occurrence or it could be driven by competitive cooperative binding facilitating the binding of unlabeled-dsDNA. In Figures 5.12 - 5.14 all the spatial frequency maps are accompanied by their spatial intensity maps to show that we did not observe local fluorescence intensity enhancements. Also, the average luminescence position, marked by the white ‘x’ in the spatial frequency maps, is centered with respect to the Atto 532 centroid position.

Figure 5.12  Reconstructed images of Atto 532 labeled dsDNA attached to AuNRs. The spatial frequency histogram maps (blue background) depict more Atto 532 emission localization on the ends of the AuNRs than at the center. The white ‘x’ in the maps represents the average location of the AuNR luminescence. The corresponding spatial intensity map (black background) for each AuNR is shown as well. The images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.
Figure 5.13  Reconstructed images of Atto 532 labeled dsDNA attached to AuNRs. The spatial frequency histogram maps (blue background) depict more Atto 532 emission localization on one end of the AuNRs than the other. The white ‘x’ in the maps represents the average location of the AuNR luminescence. The corresponding spatial intensity map (black background) for each AuNR is shown as well. The images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.

Figure 5.14  Reconstructed images of Atto 532 labeled dsDNA attached to AuNRs. The spatial frequency histogram maps (blue background) depict incomplete binding of Atto 532-dsDNA on the AuNR surface. The white ‘x’ in the maps represents the average location of the AuNR luminescence. The corresponding spatial intensity map (black background) for each AuNR is shown as well. The images share a 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.
In order to easily observe data from the reconstructed images created from Atto 532 labeled-AuNRs, we created length and width CDF plots for the data, Figure 5.15. Similar to the expectations in the TAMRA concentration study, the reconstructed image should be ~108 nm x ~50 nm. The reconstructed images with Atto 532 are still smaller than expected, except for the example pointed out in Figure 5.11, providing us with similar results as the mid concentration TAMRA-dsDNA data sets. Therefore, using a molecule with a reported longer “off” time did not fix the size mismatch issue.

Figure 5.15   Cumulative distribution function plots depicting length and width data for all reconstructed images created from imaging Atto 532 labeled-AuNR. Within a given cumulative distribution plot, each color corresponds to a different AuNR, and between the two plots each color corresponds to the same functionalized AuNR example. For the length CDF plots: Xs represent the values on the right side of the AuNR luminescence center and diamonds represent the values on the left side of center. For the width CDF plots Xs represent the values above the center and diamonds represent the values below.

5.4 INCREASING THE DISTANCE BETWEEN FLUOROPHORE AND AuNR

As a third attempt to address the size mismatch issue, we focused on the possibility of fluorophore emission coupling to the AuNR. To lower the probability of strong coupling in our initial experiments, we limited the spectral overlap between the LSPR of the AuNRs, the excitation wavelength, and the emission peak wavelength of the fluorophore. Nevertheless, there may be some overlap that can occur between the tails of the spectra, causing weak coupling. In
the event of weak coupling, the centroid position calculated for fluorophore emission will represent an intensity-weighted superposition that will be skewed towards the AuNR, reflecting the fact that some of the emission is being radiated through the AuNR plasmon modes, instead of directly from the fluorescent label. To lower the probability of fluorophore-AuNR coupling, we increased the length of the dsDNA linker.

Several research groups have conducted distance-dependence studies to find that the optimal distance between a fluorophore and a gold nanoparticle for achieving metal-enhanced fluorescence is usually around ten nanometers.\textsuperscript{16-21} Additionally, recent research from Biteen and coworkers has shown that coupling between a fluorophore and gold nanoparticle can skew the calculated centroid position of the fluorophore towards the gold nanoparticle for up to 90 nm away from the nanoparticle surface.\textsuperscript{22} However, unlike in our experiments, these experiments are usually designed to have optimal spectral overlap between the LSPR of the gold particle and the fluorophore emission. Due to this fact, we do not expect to be greatly affected by this distance-dependence; however, we wanted to test this experimentally and observe if linker distance affects the reconstructed images. To do this, we increased the length of the dsDNA linker to ~16 nm (~6.5 nm longer than the original dsDNA linker used for experiments previously). Because the enhancement field around the metallic nanoparticle decays exponentially as you move away from the surface,\textsuperscript{23-26} we hypothesized that this difference in distance should reflect some change in our reconstructed images if we were susceptible to distance-dependent coupling.

Figure 5.16-5.19 shows the reconstructed images created by mapping the location of emission from TAMRA molecules tethered to AuNRs by ~16 nm long dsDNA. For these experiments, the mid concentration of TAMRA-dsDNA was used because it supplied the best results for the TAMRA labeled-dsDNA (original dsDNA length). If we were experiencing distance-dependent coupling in our experiments, the size of the AuNRs in the reconstructed images would increase. On the contrary, the reconstructed images look similar to the mid-concentration TAMRA results tethered with the original dsDNA linker (Figures 5.3-5.6 and in Chapter 4). Also similar to previous results, we still observe binding heterogeneity based on the
localization of TAMRA molecules. The spatial frequency histogram maps (bin size is 4.4 nm x 4.4 nm for Figures 5.16-5.19) in Figure 5.16 show evenly distributed TAMRA emission localization over the AuNR surfaces, Figure 5.17 has examples displaying more localization at the ends of the AuNRs, Figure 5.18 depicts an example where more localization occurs on one end of the AuNR than the other, and Figure 5.19 includes an examples that does not yield a rod-like image. We included this example to show that we sometimes observe reconstructed images that suggest very few binding events occurred on that surface. This could be caused by cooperative binding of the unlabeled dsDNA over the labeled dsDNA, or perhaps the AuNR just randomly had less labeled-dsDNA binding.

Figure 5.16  Reconstructed images created using centroid positions calculated from emission of TAMRA tethered to AuNRs by a ~16 nm dsDNA linker. Each spatial frequency histogram map (blue background) has a corresponding intensity map (black background). The spatial frequency histogram maps show evenly distributed emission localized all over the AuNR surfaces. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.
Figure 5.17  Reconstructed images created using centroid positions calculated from emission of TAMRA tethered to AuNRs by a ~16 nm dsDNA linker. Each spatial frequency histogram map (blue background) has a corresponding intensity map (black background). The spatial frequency histogram suggests preferential binding of TAMRA-dsDNA on the AuNR ends. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.

Figure 5.18  Reconstructed images created using centroid positions calculated from emission of TAMRA tethered to AuNRs by a ~16 nm dsDNA linker. Each spatial frequency histogram map (blue background) has a corresponding intensity map (black background). The spatial frequency histogram maps suggest more binding on one end of the AuNR than the other. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar. The bin size in the reconstructed images equals 4.4 x 4.4 nm.
Figure 5.19  Reconstructed images created using centroid positions calculated from emission of TAMRA tethered to AuNRs by a ~16 nm dsDNA linker. Each spatial frequency histogram map (blue background) has a corresponding intensity map (black background). The spatial frequency histogram map suggests sparse TAMRA-dsDNA binding events on the AuNR surface. The white ‘x’ in the spatial intensity maps represents the average AuNR luminescence position for each example. All images share a common 50 nm scale bar.

The data used to create the reconstructed images in Figure 5.16-5.19 were taken in 29,192 frames, 14,596 less than what is typically used for these experiments. This is reflected in some of the spatial frequency maps where the highest frequency is lower than what was observed for the mid concentration data set in Figures 5.3-5.6. Nevertheless, even with the shorter number of frames we were still able to create rod-like reconstructed images that have the shape and orientation that match the underlying AuNR. We also included the spatial intensity maps to show that we do not observe intensity enhancements. The intensity across the AuNRs surfaces in the example shown varies by an average factor of ~4. Also, the white ‘x’ in the spatial intensity maps represents the average position of the AuNR luminescence. The luminescence is centrally located, with respect to the TAMRA emission events, as expected if this position equals the middle of the AuNR.

Finally, Figure 5.20 shows the length and width CDF plots for all the reconstructed images shown in Figure 5.16-5.19. The underlying AuNRs used in the longer dsDNA linker experiments were on average 89 nm long so we expect the reconstructed images to be ~121 nm long, after taking into account the ~16 nm linker. The data in the length CDF plot should show that 100% of the points are not reached until distances of ~53 nm on either side of the luminescence center for symmetric binding; however, the plots show that all points are
accounted for around ~30 nm from the center. We expect the width of the reconstructed images to be ~62 nm, therefore 100% of the data points should not be reached until ~31 nm from the center. Unfortunately, this happens sooner than expected at ~15 nm. Tuning the linker length did not have the desired effects; however, it is very possible that distances longer than ~16 nm are needed to truly be out of the coupling regime and see difference in the size of our reconstructed images.

Figure 5.20  Cumulative distribution function plots depicting length and width data for all reconstructed images shown in Figure 5.5. Within a given cumulative distribution plot, each color corresponds to a different AuNR, and between the two plots each color corresponds to the same functionalized AuNR example. For the length CDF plots: Xs represent the values on the right side of the AuNR luminescence center and diamonds represent the values on the left side of center. For the width CDF plots Xs represent the values above the center and diamonds represent the values below.

5.5 Conclusions

In this chapter, we described several experiments to address the size mismatch between the expected and observed dimensions of the reconstructed images of AuNRs from super-resolution imaging of fluorescently-labeled DNA coated AuNRs. These experiments included lowering the probability of having multiple molecules emitting simultaneously, as this can cause
the size of the reconstructed images to shrink relative to the actual size of the AuNR. To test this hypothesis, we varied the TAMRA-dsDNA concentration and tested a different reporter molecule that has a longer lived ‘off’ state. Unfortunately, the sizes of the reconstructed images were almost always smaller than expected. We also used a longer dsDNA linker to probe how sensitive the fluorescent tags used in the experiment are to coupling to the AuNRs. This experiment also yielded reconstructed images that were smaller than expected. One recurring trend through all the experiments was that the average luminescence position is centrally located with respect to the fluorophore centroid positions. In some cases this provided proof that the AuNR was not completely labeled with the fluorophore-dsDNA all over the length of the surface. In other cases, where the fluorophore-dsDNA bound more symmetrically, it shows that the size of the reconstructed image is being pulled towards the AuNR luminescence centroid equally in all directions. We are still in the process of understanding the cause of this size mismatch. Although we have not seen any evidence of fluorophore-AuNR coupling, such as enhanced intensity, it is possible that being near a metallic surface is altering the lifetimes and photophysics of the fluorophore. Therefore, our next step is to perform fluorescence lifetime experiments to see if there is a difference between the lifetime of free fluorophore in solution and the lifetime of fluorophore-dsDNA labeled AuNRs in solution. Also, these samples may be susceptible to forming image dipoles within the AuNR that can perturb the output point spread function, therefore skewing the centroid position calculated for the fluorophore; unfortunately, this would be difficult to correct, so it is important to rule out other contribution to this issue before focusing on the creation of image dipoles.\textsuperscript{27} Nevertheless, the reconstructed images for the different experiments continue to show the heterogeneous nature in which fluorophore-labeled dsDNA binds to AuNRs in solution.

5.6 Acknowledgements

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5.7 REFERENCES


Chapter 6: Objective-induced Point Spread Function Aberrations and their Impact on Super-resolution Microscopy

6.1 Introduction

Super-resolution optical imaging has emerged as an important strategy for overcoming the diffraction limit of light and mapping nanoscale features and processes in a variety of systems in both biology and materials science.\textsuperscript{1–12} As stated previously in Chapter 1, the simplest approach for overcoming the diffraction limit using far field imaging, involves fitting the point spread function (PSF) of a nanoscale emitter imaged through an optical system to a model function in order to localize the position of the emitter with precision of 10 nm or better.\textsuperscript{13–16} In most applications, a 2-dimensional Gaussian is used to fit the diffraction-limited emission, although this choice of function can introduce significant localization errors when imaging single dipole emitters\textsuperscript{17–19}, as we showed in Chapter 4. As a result, researchers have developed more rigorous fitting models to better describe complex emission patterns associated with dipole emitters, particularly when imaged with high numerical aperture (NA) objectives.\textsuperscript{17–20}

A second potential source of error in super-resolution imaging involves the optics used in the imaging system, which can distort the emission from a dipole source, leading to PSFs with significant aberrations from an ideal dipole emitter. As a result, many companies are developing aberration-corrected objectives focused on producing the best possible PSFs for use in demanding super-resolution imaging applications. The challenge is coming up with a test system to quantify the performance of a given optical system and determine whether the objective is producing high quality PSF images that are suitable for super-resolution imaging studies.

In Chapter 4, we successfully used the 3-dipole model to approximate the average position of stable AuNR luminescence. For this modeling approach, the AuNR luminescence was modeled as the sum of three mutually orthogonal dipole emitters and we found that it was

the better approximation model for AuNRs.\textsuperscript{18} The 3-dipole model not only produces high quality fits to the experimental PSF data associated with AuNR luminescence, but also yields several output parameters (in-plane orientation, $\phi$; out-of-plane orientation, $\theta$; and emission wavelength, $\lambda$) that can be directly compared to experimentally measured values. Thus, AuNRs provide a straightforward system to test and compare different microscope objectives to determine how the optics potentially distort the PSF and ultimately impact the quality of fits using the 3-dipole model. For this study we tested four different objectives: two Olympus UPLFLN100XOI objectives, labeled as 100x-1 and 100x-2; an Olympus PLAPON60XOTIRF, labeled as TIRF 60x; and an Olympus PLAPON60XO/PSF, labeled as PSF corrected 60x. The 100x-1 and 100x-2 objectives have an internal iris to allow for a variable NA of 0.6-1.3 (all images in this study were acquired with the iris fully open allowing for the maximum NA); the TIRF 60x has a NA of 1.45, and the PSF corrected 60x has a NA of 1.42. These objectives each have sufficient NA to collect high angle dipole emission patterns from individual AuNRs.

In this experiment we imaged single AuNRs deposited on a glass coverslip patterned with an alpha-numeric grid, allowing us to collect diffraction-limited luminescence from the same AuNR with the different objectives. This was made possible because the AuNRs are stable emitters that are not susceptible to photobleaching like fluorescent molecules.\textsuperscript{21–23} Moreover, the alpha-numeric grid allowed us to move the sample onto an atomic force microscope (AFM) to determine the orientation of each AuNR, which can be directly compared with output parameters from our fit model. This structure correlation represents another advantage of the AuNR system over fluorescent molecules, where the orientation can only be determined using polarization-dependent excitation/detection and may be sensitive to polarization distortion from the optics (such as dichroic beamsplitters).\textsuperscript{24} With this approach we were able to directly compare the differences in imaging aberrations between the four objectives and the impact each objective had in our ability to extract meaningful parameters from the 3-dipole fit.
6.2 EVALUATING GOLD NANOROD LUMINESCENCE IMAGES

Figure 6.1 shows luminescence from the same AuNR imaged with the four different objectives. The image taken with the 100x-1 objective, Figure 6.1A, shows a symmetric PSF image with a bright central feature and two oriented side lobes, as expected for the dipole-like AuNR luminescence.\textsuperscript{18,25–31} The oriented side lobes are due to high angle emission, which can only be collected using high NA objectives, and are aligned with the long axis of the nanorod, as shown by the AFM image in Figure 6.1D.\textsuperscript{26,28,30,32–34} In order to visually assess how well the 3-dipole code fit this PSF, we calculate residuals in which we subtract the 3-dipole fit from the original image. The residuals in Figure 6.1C are random and have low intensity values relative to the maximum intensity in the original image, indicating that the 3-dipole model accurately fits the symmetrical PSF emission pattern. Figure 6.1E shows the image of the same AuNR imaged with the 100x-2 objective, where the upper lobe is noticeably absent. Although the 100x-1 and 100x-2 objectives have the same model number, the images obtained using these objectives are quite different. The residuals from fitting this image with the 3-dipole model, Figure 6.1G, are non-random and are larger in magnitude than the residuals in Figure 6.1C, indicating that the 3-dipole model does not fit this data well due to the aberration introduced by the objective. The residuals in Figure 6.1G also show distinct areas of high and low magnitude (red and blue colors, respectively) in the regions where the oriented side lobes are expected to be, caused by the least squares algorithm in the code attempting to overcompensate for the missing upper lobe.
Figure 6.1  (First column: A, E, I, and M) AuNR luminescence taken with the 100x-1, 100x-2, TIRF 60x, and PSF corrected 60x objectives, respectively. (Second column: B, F, J, and N) 3-dipole fit to the corresponding image from each of the four objectives. (Third column: C, G, K, and O) Residuals (image – fit) for each image taken with the different objectives. (Fourth column: D, H, L, and P) The correlated AFM image of the AuNR (100 nm scale bars). The black line on each AFM image represents the calculated in-plane angle, \( \phi \), from the 3-dipole fit to each image taken with the different objectives. The arrowhead is pointing in the direction of the fit angle. Images A-C share a common 500 nm scale bar, images E-G share a common 500 nm scale bar, and images I-K and M-O share a common 500 nm scale bar. The color map bars indicate intensity values.

The AuNR luminescence images using the TIRF 60x and PSF corrected 60x, Figure 6.1I and Figure 6.1M respectively, also exhibit asymmetric patterns in the AuNR PSF. For these objectives, the patterns can be somewhat more difficult to see due to the lower overall magnification. However, the asymmetry in the images can be confirmed in the non-random residuals, Figures 6.1K and 6.11O, although the magnitude of the residuals of these fits relative to the peak intensity is lower compared to the 100x-2 objective. Despite the NA values of both the TIRF 60x and PSF corrected 60x being greater than 1.3, the objectives are not able to
symmetrically collect the light emitted at high angles from the AuNRs, resulting in asymmetric PSFs.

In all four AFM images shown in Figure 6.1 (panels D, H, L, P), we include the calculated value of the in-plane angle, \( \phi_{\text{fit}} \), which is the projection of the long axis of the nanorod into the x-y sample plane and can have values between 0° and 360° (Figure 6.2). By comparing the value of an output parameter from the fits to an experimental measurable, we have a standard by which we can evaluate the quality of the fit. Because of the symmetry of the AuNRs, a value of \( \phi \) and \( \phi \pm 180° \) should be identical for a planar AuNR (out-of-plane angle (\( \theta \)) = 90°, Figure 6.2); however, an out-of-plane tilt (\( \theta \neq 90° \)) will break this symmetry leading to a single correct value of \( \phi \). We have previously reported that we fit each AuNR twice using \( \phi \) and \( \phi \pm 180° \) as the initial guesses and then choose the correct value of \( \phi_{\text{fit}} \) based on the value of the out-of-plane angle \( \theta_{\text{fit}} \). For example, the out-of-plane angle (\( \theta_{\text{fit},1} \)) for the AuNR shown in Figure 6.1A is 77° and that corresponds to a calculated \( \phi_{\text{fit},1} \) value of 47° which aligns well with the AFM image of the AuNR (Figure 6.1D). On the other hand, the value for \( \phi_{\text{fit},2} \) was 227° with a \( \theta_{\text{fit},2} \) of 90°. The fitting code sets the upper limit on \( \theta_{\text{fit}} \) to be 90°; therefore, calculating a value of \( \theta \) greater than 90° is not possible. For symmetry to be preserved, a rod with \( \phi = 47° \) and \( \theta = 77° \) would be equivalent to a rod with \( \phi = 227° \) and \( \theta = 103° \); however, the code will not allow this, and so it reaches the upper bound of \( \theta_{\text{fit}} = 90° \) when \( \phi = 227° \). Based on this analysis, we then know that the “true” value of \( \phi_{\text{fit}} \) is the value at which \( \theta_{\text{fit}} \neq 90° \). This is true except for the rare case when the rod is truly planar, and both values of \( \phi \) yield \( \theta_{\text{fit}} = 90° \). Qualitatively, we observe the best agreement between \( \phi_{\text{fit}} \) and the orientation of the long axis of the AuNR for 100x-1, followed by 100x-2. The TIRF 60x calculates a \( \phi_{\text{fit}} \) value that is 180° away from the other three objectives. In all four cases, the fits produce a slight out-of-plane tilt (\( \theta \neq 90° \)), suggesting that the TIRF 60x yields the incorrect value of \( \phi_{\text{fit}} \).
Figure 6.2  Diagram depicting the in-plane angle, $\phi$, of the AuNR in the x-y sample plane. The out-of-plane angle, $\theta$, is also shown. Both $\phi$ and $\theta$ correspond to the long axis of the AuNR and $\theta = 90^\circ$ corresponds to planar AuNR.

Figure 6.3 shows similar results for a second AuNR oriented at a different angle. At this new orientation, the 100x-1 objective, which produced the best results in Figure 6.1, introduces a slight asymmetric imaging aberration to the collected emission pattern, shown in Figure 6.3A. The aberration is more pronounced in the fit residuals, Figure 6.3C, that are non-random and have a higher magnitude relative to the peak intensity compared to Figure 6.1C. Although the 100x-1 introduces some aberration with this particular AuNR orientation, it still produces the best image of the objectives tested based on the residuals. The fit residuals for the data collected with the other objectives, Figures 6.3G, 6.3K, and 6.3O are also non-random and show significant systematic features.
Figure 6.3 (First column: A, E, I, and M) AuNR luminescence taken with the 100x-1, 100x-2, TIRF 60x, and PSF corrected 60x objectives, respectively. (Second column: B, F, J, and N) 3-dipole fit to the corresponding image from each of the four objectives. (Third column: C, G, K, and O) Residuals (image – fit) for each image taken with the different objectives. (Fourth column: D, H, L, and P) The correlated AFM image of the AuNR (100 nm scale bars). The black line on each AFM image represents the calculated in-plane angle, $\phi_{fit}$, from the 3-dipole fit to each image taken with the different objectives. The arrowhead is pointing in the direction of the fit angle. Images A-C share a common 500 nm scale bar, images E-F share a common 500 nm scale bar, images I-K share a common 500 nm scale bar, and images M-O share a common 500 nm scale bar. The color map bars indicate intensity values.

As before, we plot the calculated in-plane angle, $\phi_{fit}$, on top of the AFM images in Figure 6.3 (panels D, H, L, P). In this case, the 100x-1, TIRF 60x, and PSF corrected 60x all show excellent agreement, both with the measured orientation of the AuNR and with each other. The 100x-2 objective, on the other hand, calculates a flipped value of $\phi_{fit}$ that does not lie perfectly along the long axis of the AuNR, indicating that this objective yields an incorrect fit. For this AuNR, we also measured the dark field scattering spectrum and used the peak wavelength to compare with the values of $\lambda_{fit}$ calculated by the 3-dipole fit. Previous work in this field has shown that the luminescence maximum and the maximum taken from dark field scattering
spectra are equal and therefore, can be directly compared. The experimental peak in the dark field scattering spectrum was at 622 nm, while the 100x-1, 100x-2, TIRF 60x, and PSF corrected 60x produced $\lambda_{\text{fit}}$ values of 662, 693, 724, and 686 nm, respectively. In previous work from our group, we reported that $\lambda_{\text{fit}}$ is consistently red shifted relative to the experimental value, most likely due to the fact that the image consists of multiple wavelengths, which is not accounted for in the 3-dipole fit. Nonetheless, we find that the 100x-1 objective produces a $\lambda_{\text{fit}}$ value closest to the experimental value, as well as fits with the most random residuals and most consistent agreement with experimental values of $\phi$; as a result, we will use this objective as a benchmark when determining the correct orientation of the measured value of $\phi (\phi_{\text{meas}})$ in subsequent studies.

### 6.3 Comparing 3-dipole Output Parameters to Experimental Values

To further analyze how the 3-dipole output parameters compare with experimental values, we imaged multiple AuNRs with the four objectives. Figure 6.4A compares different $\Delta \phi_{(\text{fit-mea})} = \phi_{\text{fit}} - \phi_{\text{meas}}$ for the data taken with the four objectives. $\phi_{\text{meas}}$ was determined from the AFM image by measuring the angle of the AuNR along its long axis with the built-in angle measuring tool in the AFM software. Figure 6.4A shows data for 26 AuNRs imaged with the 100x-1, 100x-2, and TIRF 60x objectives. Only 9 of those 26 AuNRs were imaged with the PSF corrected 60x, due to the limited time the objective was on loan to the lab. The 100x-1 histogram of $\Delta \phi_{(\text{fit-mea})}$ shows values clustered around ~$0^\circ$ (as expected since this objective was used to assign the correct orientation of $\phi_{\text{meas}}$) with a standard deviation of 4º. The 100x-2, TIRF 60x, and PSF corrected 60x histograms in Figure 6.4A, on the other hand, show occurrences of $\Delta \phi_{(\text{fit-mea})}$ clustered around ~$0^\circ$ and ~$180^\circ$, indicating that $\phi_{\text{fit}}$ is flipped in many of the fits (particularly for the 100x-2 objective). The TIRF 60x and PSF corrected 60x histograms also show occurrences of $\Delta \phi_{(\text{fit-mea})}$ around ~$360^\circ$, but by symmetry, this is not a problem. The TIRF 60x histogram does include an event for $\Delta \phi_{(\text{fit-mea})}$ of ~$90^\circ$, which indicates a very poor fit to the experimental data.
Figure 6.4  (A) Histograms for data collected with each objective showing the differences between the calculated in-plane angle, $\phi_{\text{fit}}$, and the measured in-plane angle, $\phi_{\text{meas}}$. (B) Histograms for data collected with each objective showing the differences between the calculated luminescence peak maximum, $\lambda_{\text{fit}}$, and the experimental dark field scattering maximum, $\lambda_{\text{meas}}$. The y-axis on all the histograms represents the frequency of each occurrence.

Figure 6.4B compares the calculated AuNR luminescence intensity peak maximum, $\lambda_{\text{fit}}$, to the peak maximum obtained from the dark field scattering spectra, $\lambda_{\text{meas}}$, for each AuNR. The data for 20 AuNRs were included in this figure; only 8 of those 20 were imaged with the PSF.
corrected 60x objective. The 100x-1, 100x-2, and PSF corrected 60x histograms show the majority of occurrences for $\Delta \lambda_{(\text{fit - measured})}$ between $\sim 35$-$60$ nm, whereas the TIRF 60x histograms shows the majority of clustering between $\Delta \lambda_{(\text{fit - measured})}$ values of $\sim 15$-$35$ nm. The overall conclusion from Figure 6.4B is that $\lambda_{\text{fit}}$ is always over-estimated by the 3-dipole fit model, in agreement with previous results.\(^{18}\)

![Figure 6.5](image)

Figure 6.5  Comparison of the difference in the calculated centroid positions (calculated using the 3-dipole model and a 2-D Gaussian model) to calculated out-of plane angle, $\theta_{\text{fit}}$. The pink dots represent data collected with the 100x-1 objective, the green triangles represent the 100x-2 objective data, the blue stars represent the TIRF 60x objective data, and the red crosses represent the PSF corrected 60x objective data. The line of best fit, dotted dark gray line, from a previous study\(^{18}\) was included as a comparison.

In previous work from our research group and others it was noted that the difference between centroid positions of a dipole emitter, calculated using either the 2-dimensional Gaussian or dipole emission model, would increase as the out-of-plane angle of the dipole emitter increased.\(^{17,18,35}\) To test if the different objectives followed this expected trend, the output centroid position from the 3-dipole model was compared to the centroid position calculated when the AuNR is fit to a 2-dimensional Gaussian (Equation 1). Where $I$ is the spatially-dependent intensity of the emission spot, $z_0$ is the intensity of the background, $I_0$ is the peak intensity of the
emission, \( s_x \) and \( s_y \) are the widths of the Gaussian in \( x \) and \( y \), and the centroid position is \( x_0 \) and \( y_0 \).

\[
I(x,y) = z_0 + I_0 e^{-\left[\frac{1}{2} \left( \frac{x-x_0}{s_x} \right)^2 + \frac{1}{2} \left( \frac{y-y_0}{s_y} \right)^2 \right]}
\]  

(1)

The distance between the two centroid position points, \( \Delta_{\text{dipole-Gaussian}} \), was calculated using Equation 2, where \((x_2, y_2)\) correspond to the centroid position calculated using a 2-Dimensional Gaussian and \((x_1, y_1)\) correspond to the centroid position calculated using the 3-dipole model. The distance difference was plotted against the out-of-plane angle, \( \theta_{\text{fit}} \), from the 3-dipole model (Figure 6.5).

\[
\Delta_{\text{dipole-Gaussian}} = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}
\]  

(2)

The dark gray dotted line in Figure 6.5 represents the line of best fit from the previously reported results using the 100x-1 objective and is included for direct comparison.\(^1\) The same AuNRs from Figure 6.4A were used in Figure 6.5. The \( \Delta_{\text{dipole-Gaussian}} \) values obtained from images taken with the 100x-1 and PSF corrected 60x objectives follow the trend of increasing distances between the calculated centroid positions as \( \theta_{\text{fit}} \) increases, tracking well with the line of best fit. The values obtained from images taken with the 100x-2 also track well with the previous result; however, there is one outlier with a \( \theta_{\text{fit}} \) of \( \sim 79^\circ \) and a \( \Delta_{\text{dipole-Gaussian}} \) of \( \sim 2.4 \) nm that is contrary to the expected trend. The \( \Delta_{\text{dipole-Gaussian}} \) values obtained from images taken with the TIRF 60x show higher distances between the two centroid position points even when the AuNR is planar; therefore, the TIRF 60x results differed the most from expected results. Moreover, this objective yielded many \( \theta_{\text{fit}} \) values below \( 85^\circ \), in contrast to the other three objectives, indicating that the aberrations caused by this objective yield an artificial PSF that makes the AuNR seem well out-of-plane from the substrate.

6.4 Conclusions

Despite the fact that the different objectives introduced significant aberrations to the PSF of the AuNRs, which could be observed even by eye, we were surprised to discover that the
output parameters did not differ significantly across the different data sets. While the values of \( \phi_{\text{fit}} \) were sometimes flipped relative to the benchmark 100x-1 objective, they were typically \( \pm 180^\circ \) which is consistent with the high degree of symmetry and relative planarity of the AuNRs studied. The values of \( \lambda_{\text{fit}} \) were also fairly clustered for the different objectives, indicating that the lower quality PSFs generated by several of the objectives had minimal impact on the majority of the fits. Even when comparing the distance between the centroid positions determined by two different models, we found that three of the four objectives produced fairly similar results for most AuNRs studied, suggesting that the centroid positions would have similar values. Unfortunately, we could not directly compare centroid positions calculated by each of the four objectives, since the samples were moved between the different experiments, and drift within the system has limited our sample registration to \( \sim 50 \) nm at best. In principle, we could further characterize the super-resolution ability of these objectives by attempting to resolve two AuNRs spaced by less than the diffraction limit, although this presents significant obstacles because the gold does not bleach or blink, and we must be concerned about plasmon coupling effects. However, by using other output parameters generated by the 3-dipole fit, including the in-plane orientation and the peak emission wavelength, we have a straightforward means of comparing quality of fit which provides insight into how well the different objectives will perform in super-resolution studies.

In these studies, the TIRF 60x produced the most consistent problems, with significant variation in \( \phi_{\text{fit}} \), much lower values of \( \theta_{\text{fit}} \), and the most scatter in the centroid calculation data; as such, we would not suggest using our particular TIRF 60x objective for super-resolution imaging studies. We also observe that objectives with the same model number can offer different performance: the 100x-1 objective provides the most consistent quality of fits and experimental agreement with output parameters, while the 100x-2 objective has more consistent aberrations that are visible by eye. Thus, it is important to test individual objectives to ensure their utility for super-resolution imaging. The origin of this effect remains unknown, although we have speculated that the internal iris in the variable NA objective may be misaligned leading to some
of the high angle light being cut off. Fourier plane imaging could help determine whether this hypothesis is true. We also point out that the aberrations are present across the entire wide-field image, indicating that this is not a position-dependent effect. Despite several of the problems described above, we find that in many cases the four objectives yield similar output parameters, indicating that only certain AuNRs (typically those with a particular orientation) have strong aberrations in the PSF that lead to significant deviations in the output parameters. Thus, it is important to test a wide data set across multiple AuNR orientations to characterize the performance of a particular objective. AuNRs provide an excellent test system for characterizing objective-induced PSF aberrations by providing a simple, stable platform with directly measurable experimental values that can be compared to variables fit using a 3-dipole model.

6.5 ACKNOWLEDGEMENTS

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6.6 REFERENCES


Chapter 7: Conclusions and Future Experiments

7.1 CONCLUSIONS

The experiments and results discussed in this dissertation were focused on mapping the location of fluorescently-labeled dsDNA bound to AuNRs. We used a triplet-state mediated technique to photoswitch the fluorophores between emissive and non-emissive states. The photons detected from a fluorophore in the emissive state was fit to a two-dimensional Gaussian to extract the location from which the emission originated. The location of the emission was used to map the apparent location of where an individual dsDNA was bound. With this technique we were able to observe differences in the locations of where dsDNA binds to AuNRs over many samples and we hypothesized that the differences were caused by the apparent binding heterogeneity of labeled-dsDNA on AuNRs surface.

Using the centroid positions for the fluorophores attached to AuNRs we were able to create reconstructed images that yielded the right shape and orientation of the underlying AuNR. Unfortunately, the size of the reconstructed AuNR image was almost always smaller than expected. If we were mapping the true location of the dsDNA then the reconstructed AuNR should have the expected AuNR dimensions. Nevertheless, we believe that the size mismatch did not affect our observation of the binding heterogeneity. We tested several hypotheses to address this issue; first we started using a better fit model to approximate the AuNR luminescence contribution to avoid skewing the fluorophore centroid position towards the luminescence position. Second, we altered the concentration of dsDNA available to bind to the AuNRs in solution. We changed the labeling fluorophore to use a molecule with a longer lived non-emissive state. The latter two experiments aimed at lowering the probability of multiple fluorophores emitting simultaneously, which would result in a calculated centroid position that reflects an intensity-weighted super-position and cause a smaller than expected reconstructed image size. Finally, we used a longer dsDNA linker to space the fluorophore from the AuNR surface. These approaches did not solve the size mismatch issue; fortunately, we still have other
hypotheses to test. Moreover, by solving this issue we hope to increase the applications for which this technique may be applied.

7.2 Future Experiments

Solving Size Mismatch

We have hypothesized that plasmon coupling between the fluorescent molecule and the AuNR is potentially a huge factor in why the reconstructed images are smaller than expected. If strong plasmon coupling is occurring, then instead of emitting into the far-field (Figure 7.1A) the fluorophore emission is coupled into the AuNR which provides other pathways for emission (Figure 7.1B).\textsuperscript{1-5} Therefore, the centroid position of that coupled emission will overlap with the centroid position of the AuNR, effectively shrinking the reconstructed size. In the experiments discussed in this dissertation we attempted to minimize spectral overlap between the AuNR localized surface plasmon resonance (LSPR), the fluorophore excitation and emission, and the laser excitation source. In doing this we hoped to minimize the probability that strong plasmon coupling would occur; however, we were still susceptible to weaker coupling where the calculated centroid position of the fluorophore may be intensity-weighted and skewed towards the AuNR.

![Figure 7.1](image)

Figure 7.1 Depiction of how plasmon coupling affects the calculated centroid position (blue x) of the fluorophore. (A) The fluorophore emits into the far-field and the calculated centroid position represents the true position of the molecule. (B) Strong fluorophore-AuNR plasmon coupling skews the fluorophore centroid position towards the AuNR. (C) Weak coupling results in an intensity-weighted skewed centroid position.
To further minimize plasmon coupling we can tune several different experimental parameters. First, we can use longer AuNRs that have a LSPR in the near-IR wavelength range to ensure that there is no overlap between the LSPR and the excitation/emission of the fluorophore. By using this approach we limit the probability of strong and weak coupling occurring. Long AuNRs, prepared chemically,\textsuperscript{6} will be functionalized using the technique discussed in Chapter 2. Ideally, by eliminating the probability of plasmon coupling, the calculated centroid positions should represent the true position of the dsDNA. A second approach involves using a different spacer layer to increase the distance between the fluorophore and the AuNR surface. To do this we plan on using a silica layer rather than dsDNA because the rigidity of the dsDNA at lengths longer than what was discussed in this dissertation is a worrying point. Our functionalization strategy would need to be altered as the fluorophore would be attached to the silica surface and this is typically done \textit{via} azide linking reactions.\textsuperscript{5,7,8} By increasing the distance between the AuNR and the fluorophore we decrease the probability of coupling interactions.\textsuperscript{5,9–11} Also, this strategy is easily applicable to the longer AuNRs and the size of AuNRs that were discussed in this dissertation.

\textbf{Incorporating Different Ligands and Nanoparticles}

The experiments in this dissertation all discussed using dsDNA as the ligand of interest. Moving forward with future experiments, we would like to apply this technique to study how different fluorescently-labeled ligands bind to nanoparticles in solution. We could test the probability, over many single particles, of molecules such as biotin or cucurbiturils binding preferentially to the ends of AuNRs and if the addition of other ligands affects that outcome.\textsuperscript{12,13} Larger molecules, such as proteins, would also be useful ligands to study as they would provide a larger footprint on the AuNR surface than dsDNA. By covering more surface with a protein we limit the amount of fluorophores on the surface available to emit simultaneously. It would also be interesting to observe any correlations between ligand size and binding location on the nanoparticle surface. Moreover, after fully understanding this technique with using AuNR as the
substrate, moving on to different nanoparticle shapes is also a possibility. Gold nanospheres, for instance, are of interest because they are often used in multifunctional nanoparticle experiments.\textsuperscript{14–18} Understanding how ligands bind to isotropic structures may provide insight that was not gained in using anisotropic structures.

**Investigating Cooperative Binding**

We would also like to investigate the extent to which cooperative binding affects the locations of where ligands bind. Doing this would involve using identical ligands labeled with different reporter molecules, to create two different ligand populations, and two excitation sources for both of the fluorophores. Also, gold nanowires or a thick silica shell will be needed to limit plasmonic coupling depending on which fluorophores were chosen. With this experimental setup we may observe how the binding of one type of ligand will facilitate the binding of that same ligand, so the results may show one ligand binding primarily to certain regions of the particle over the second ligand. On the contrary, it is possible that we may also observe an even mixture of the two ligands binding across the particle surface. This experiment would also allow us to gain insight on how the initial stoichiometry of the two ligands in solution affects the binding ratio on the particle surface. It has been previously reported that the stoichiometry in solution is not retained on the particle surface and this approach would allow us to see if the ratio is different or the same over many single particles.\textsuperscript{19} The results of these experiments would be very applicable to experiments involving multifunctional nanoparticles.

**7.3 References**


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