The development of optical microscopy techniques for the advancement of single-particle studies

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ABSTRACT

Single particle orientation and rotational tracking (SPORT) has recently become a powerful optical microscopy tool that can expose many molecular motions. Unfortunately, there is not yet a single microscopy technique that can decipher all particle motions in all environmental conditions, thus there are limitations to current technologies. Within, the two powerful microscopy tools of total internal reflection and interferometry are advanced to determine the position, orientation, and optical properties of metallic nanoparticles in a variety of environments.

Total internal reflection is an optical phenomenon that has been applied to microscopy to produce either fluorescent or scattered light. The non-invasive far-field imaging technique is coupled with a near-field illumination scheme that allows for better axial resolution than confocal microscopy and epi-fluorescence microscopy. By controlling the incident illumination angle using total internal reflection fluorescence (TIRF) microscopy, a new type of imaging probe called “non-blinking” quantum dots (NBQDs) were super-localized in the axial direction to sub-10-nm precision. These particles were also used to study the rotational motion of microtubules being propelled by the motor protein kinesin across the substrate surface.

The same instrument was modified to function under total internal reflection scattering (TIRS) microscopy to study metallic anisotropic nanoparticles and their dynamic interactions with synthetic lipid bilayers. Utilizing two illumination lasers with opposite polarization directions at wavelengths corresponding to the short and long axis surface plasmon resonance (SPR) of the nanoparticles, both the in-plane and out-of-plane movements of many particles could be tracked simultaneously. When combined with Gaussian point spread function (PSF) fitting for particle super-localization, the binding status and rotational movement could be resolved without degeneracy.

TIRS microscopy was also used to find the 3D orientation of stationary metallic anisotropic nanoparticles utilizing only long-axis SPR enhancement. The polarization direction of the illuminating light was rotated causing the relative intensity of p-polarized and s-polarized light within the evanescent field to change. The interaction of the evanescent field with the particles is dependent on the orientation of the particle producing an intensity curve. This curve

and the in-plane angle can be compared with simulations to accurately determine the 3D orientation.

Differential interference contrast (DIC) microscopy is another non-invasive far-field technique based upon interferometry that does not rely on staining or other contrast enhancing techniques. In addition, high numerical aperture condensers and objectives can be used to give a very narrow depth of field allowing for the optical tomography of samples, which makes it an ideal candidate to study biological systems. DIC microscopy has also proven itself in determining the orientation of gold nanorods in both engineered environments and within cells.

Many types of nanoparticles and nanostructures have been synthesized using lithographic techniques on silicon wafer substrates. Traditionally, reflective mode DIC microscopes have been developed and applied to the topographical study of reflective substrates and the imaging of chips on silicon wafers. Herein, a laser-illuminated reflected-mode DIC was developed for studying nanoparticles on reflective surfaces.

CHAPTER 1: GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized in a manner similar to a scientific peer-reviewed journal article. There is an introduction of the pertinent background followed by the scientific investigations and the resulting conclusions. Within each chapter are the necessary cited literature, figures, and tables.

The first chapter is a general introduction that gives a brief overview of microscope development followed by the introduction of the three optical microscope configurations used for the majority of the research presented within. These configurations include total internal reflection fluorescence (TIRF) microscopy, total internal reflection scattering (TIRS) microscopy, and differential interference contrast (DIC) microscopy.

The second chapter describes the integration of a new type of fluorescent probe into super-localization and high-precision tracking through optical microscopy. The probe is used to investigate the high interest motor protein kinesin within a microtubule gliding assay.

Chapter three is dedicated to the introduction of a new optical microscopy technique that can track the dynamic three-dimensional (3D) rotational movement of anisotropic nanoparticles while simultaneously super-localizing their lateral position. Gold nanorods were studied interacting with a phospholipid bilayer surface.

The determination of the 3D orientation for stationary anisotropic nanoparticles using TIRS microscopy is described in Chapter 4. Instead of using two-color illumination with opposing polarizations, a single wavelength technique is explored.

The description of a bench top laser-illuminated reflected-mode DIC can be found in chapter five. Nanoparticles and nanostructures on reflective surfaces were imaged giving insight into lithographic modes for nanoparticle synthesis. The system is also capable of long-term observation for dynamic processes.

The final chapter summarizes the content of the thesis and provides insight into possible avenues for future research.

Introduction

Of the five senses humans perceive, sight is often heralded as the most magnificent; the one we would last like to live without. With our eyes we are able to observe our surroundings, and with those observations we can start to investigate natural phenomena. From birth our natural instincts are to start making sense of the universe we live in. This starts with the most basic observations; what is around the corner, what is outside this room? Eventually our curiosity leads us to peering up into space straining to see the farthest out we can, or staring down into our world, squinting trying to resolve the most detail we can. Though they are amazing products of evolution, our eyes have significant limitations.

Our eyes are limited to detecting a very small region of the electromagnetic spectrum we called the "visible region" consisting of the wavelengths between 400 nm and 700 nm. To overcome this limitation we developed sensors to collect information outside this region that can translate it into something we can comprehend. We are also limited on our ability to resolve objects that are far away and very small. For this limitation we have developed optics to aid us such as telescopes and microscopes.

Although there are three main modern types of microscopy (optical,[1] electron,[2] and scanning probe microscopy[3]), optical microscopy is what commonly comes to mind first. The invention of the first compound microscope (consisting of two or more lenses) has some controversy surrounding it, but is generally given to Zacharias Janssen (1580-1638) of the Netherlands around the year of 1595.[4]

Early microscopes suffered from two significant limitations typically referred to as aberrations.[5] The first is what is called the spherical aberration, which results from the axial and peripheral rays having different focal planes. The second is chromatic aberration, which is due to the extent of refraction from different wavelengths of light. Both of these aberrations result in the blurring of the image and the loss of resolution.

It was not until the German mechanic Carl Zeiss (1816-1888) started producing microscopes in the late 19th century that these limitations were overcome.[5] At the time, producing quality optics was performed by trial and error. To establish a more reliable method, Carl Zeiss employed the mathematician and physicist Ernst Abbe who eventually came up with the original formula for the calculation of the maximal resolution achievable from an optical microscope. The formula for resolution d is seen in equation 1 below

$$d = \frac{1.22\lambda}{NA_{obj} + NA_{cond}} \quad (1)$$

where λ is the wavelength of the light, NA_{obj} is the numerical aperture of the objective, and NA_{cond} is the numerical aperture of the condenser. Numerical aperture is defined as the refractive index of the imaging medium (n) multiplied by the sine of the aperture angle (α): N.A.= $n \cdot \sin(\alpha)$.

While there are many types of optical techniques including bright field, dark field, phase contrast, *etc*.[1] the majority of this dissertation focuses around the technique of using totally internally reflected light to produce either fluorescent light or scattered light that is imaged by the objective.

Total internal reflection fluorescence microscopy (TIRFM) is an optical sectioning technique that has excelled in the study of molecular dynamics at solid/liquid interfaces and the study of cellular organization and dynamic processes within and near cellular membranes. Light propagating through a transparent medium will undergo total internal reflection (TIR) when it encounters an interface of a second medium with a lower index of refraction at an angle greater than the critical angle (θ c) (from the normal of the interface). When TIR occurs, an evanescent field (EF) is generated at the interface of the two media characteristic of the reflected light beam that exponentially decays as distance increases from the surface. This EF can be used to excite fluorophores at a distance of a few hundred nanometers from the interface while essentially eliminating the out-of-focus fluorescence background.

After nearly three decades of intense research, TIRFM has already morphed into a mature technique for biological imaging by the time Axelrod published his last comprehensive review on TIRFM in 2008.[6] In this section, we will focus on the applications and techniques pertinent to single cell imaging published since 2008.

Recent Advances in Instrumentation

Automated Prism-Based System for High-Precision Imaging. There are two basic types of TIRFM as determined by the optics that produce TIR. The first is objective-based TIRFM where the laser beam is directed off-center down a high NA objective. The optics within the objective produce a reflected beam at an angle equal to or greater than the critical angle, and TIR occurs at

the coverslip/sample interface. The emission signal is then directed back through the objective to the signal recorder. The second type of TIRFM is prism-based. Laser illumination is directed through the prism on which the sample lies. TIR occurs at the coverslip/sample interface, and emission is collected by an objective located on the opposite side of the prism. Various configurations of these two types of TIRFM have been discussed in a previous review.[6]

Each type of TIRFM holds its own advantages and drawbacks. The objective-based TIRFM is compact and commercially available as a module for standard light microscopes. Its main drawbacks include excitation light scattered within the objective, the difficulty in determining the incident angle, and the limitation on the range of achievable incident angles due to the geometry of the objective. These drawbacks can negatively influence the detection sensitivity and axial localization precision of fluorescent probes. All of these drawbacks can be avoided in prism-based TIRFM, which makes it an attractive technique for high-precision tracking applications. However, the performance of the prism-based system largely depends on the accuracy, precision, and reproducibility of the tedious, time-consuming calibration procedure to find the ideal illumination conditions at different incident angles.

To harvest the full benefits of the prism-based TIRFM and reduce the burden on the operator, an automated prism-based TIRFM was developed recently with the capability to accurately determine the ideal illumination conditions for a wide range of angles.[7] Once calibrated, the system can scan reliably and reproducibly through a wide range of incident angles with intervals as small as 0.1°. The unbiased calibration procedure ensures that the measured fluorescence intensities at tens to over a hundred different incident angles are consistent so that the data sets can be nonlinear least-squares fit with the decay functions to achieve high precision axial localization and better practical axial resolution.[7] It should be pointed out that this improvement is only achievable with a homogeneous liquid sample above the TIR surface. For a heterogeneous sample, such as cells, there is still no good way of accurately measuring the local EF field depth and profile.

Combined with the continuous fluorescent emission from nonblinking QDs,[8] the automated TIRFM can locate and track events taking place within the EF with exceptionally high precision.[9] The use of nonblinking QDs is necessary to avoid erratic fluorescence intenstiy curves due to conventional fluorescent probes' tendency to blink during system calibration and data acquisition. The axial distances of nonblinking QDs attached to stationary microtubules can

thus be determined with sub-10 nm precision, and the rotation of microtubules driven by kinesin motors can be detected in real time by resolving the movement of nonblinking QDs within a small vertical distance of ~50 nm near the surface.[9]

Using a similar variable angle approach, Yang et al. reconstructed 3D microtubules within PtK2 cells using a Bayesian framework and quantified the lateral and axial curvatures of single microtubules by comparing their data to the computer simulations and electron microscopy images.[10]

New Illumination Schemes. The EF generated in TIRFM is no more than a few hundred nanometers in depth at the interface, which has limited the applicability of TIRFM to biological imaging. To work around this hindrance, the strategy of imaging at subcritical angles that are smaller than yet still close to the critical angle was proposed. At a subcritical incident angle, the excitation laser beam is refracted to produce a slanted illumination path; thus, it is possible to extend the thin illumination layer several micrometers into the cell body. The narrow field of illumination results in higher S/N than epi-fluorescence microscopy. This technique was coined variable-angle epi-fluorescence microscopy (VAEM),[11] highly inclined thin illumination (HILO),[12] or simply known as pseudo-TIRFM. The emitted light as a consequence of angled illumination, if collected directly, would appear tilted at the angle the sample is illuminated. By using a series of objectives and additional optics, oblique plane microscopy (OPM) can translate the image to be collected "flat" on the CCD.[13] All of these early implementations of pseudo-TIRFM were objective-based (Figure 1B). More recently, the same automation strategy described in the previous section was employed for prism-based pseudo-TIRFM (Figure 1D).[14]

Another improvement on illumination scheme was intended to remove the effect of interference fringes at different incident angles. The intensity profile of the incident laser can be negatively affected by scattering in the imperfect light path to give rise to interference fringes, resulting in a nonuniform illumination of the sample. Built upon the idea of azimuthal spinning of the incident laser beam,[15] Fiolka et al. used a piezo mirror to conveniently control the incident angle while producing an even sample illumination.[16]

In yet another effort to obtain both high S/N offered by prism-based TIRFM and the versatility of objective-based TIRFM in choosing thick sample substrates such as perfusion chambers and microarrays, a lightguide (LG)-based TIRFM has been constructed that bypasses excitation/emission interference while allowing applications requiring large sample holders and

large uniform evanescent fields.[17] Multicolor LG-TIRFM has been demonstrated for tracking dynamic lipids rafts on living cells cultured in perfusion chambers.[17] The fixed incident angle is considered a major drawback of LG-TIRFM.

New Substrates. Typical microscope slides and coverslips are usually chosen as the sample substrate for cell imaging because they allow for TIR and cell adhesion to the surface. Unfortunately, this can limit chemical access to the cell membrane due to cell surface contact. By using silica colloidal crystals as a porous substrate, researchers were able to allow ligand access to the cell membrane while still producing TIR angles in a wide range.[18] In another study, by changing the substrate to which the cells adhered to a subwavelength nanograting, fluorescence detection sensitivity was improved by coupled plasmon excitation.[19]

Integration with Other Techniques. To selectively monitor the dynamics between membrane bound proteins and a functionalized surface, a combination of TIRFM and optical trap was developed to "drop" a cell onto the surface under the objective.[20] This trap allows precise control of the initiation of interactions between a cell and a surface of interest, while the TIRFM could continuously monitor the surface interaction from the moment on onset. While an optical trap may be useful for single cell analysis, sometimes a high-throughput device is wanted for examining large batches of cells. To test the heterogeneity in a population of cells, TIRFM was combined with flow cytometry to examine cells at rates of 100−150 cells/s with single cell resolution.[21] The hydrodynamic focusing of the cells to the objective-based TIRFM allowed for the high-throughput sorting of cells based on their fluorescent signal. This signal can help determine how a large population of cells responds to certain conditions.

Super-Resolution under TIRFM. The intrinsic background reduction and high accessibility found in TIRFM make it a quality stepping point for super-resolution techniques. In stochastic optical reconstruction microscopy (STORM) or photoactivated localization microscopy (PALM), the decreased background associated with the optical sectioning allows for localization of the stochastically blinking fluorophores with fewer recorded photons than other wide-field methods. A prism-based setup also allows for the easy integration of multiple laser lines needed for the excitation and activation of the fluorophores, making STORM or PALM an accessible method for those needing to improve the lateral resolution in TIRFM.

A stimulated emission depletion (STED) microscope setup has been coupled to a TIRFM.[22] The advantage of this integration is that the STED system provides subdiffraction

lateral resolution while TIRFM limits the illumination depth, allowing for optical tomography. The authors were able to image immuno-stained microtubules within PtK2 cells at STED resolution while minimizing the penetration depth of the illumination source, thus reducing photo bleaching and phototoxicity.

Structured illumination microscopy (SIM) has also been coupled with TIRFM for the imaging of single cells in the past few years.[23-26] The easy integration with an inverted objective-based TIRF microscope allows for increased accessibility for researchers. While the resolution is not as good as stochastic techniques or STED, SIM-TIRFM has been able to break the 100 nm resolution barrier, and with the addition of a ferroelectric liquid crystal on silicon spatial light modulator, it is now possible to take images at video rate.[23]

Recent Applications in Membrane Studies and Plant Cell Imaging

Membrane Studies. While the variability within the TIRFM technique is considered a reason for its successful implementation in many studies, simple unadulterated TIRFM can reveal much information about cellular membrane processes. Recent membrane investigations include the use of TIRFM to document real-time trafficking of a dopamine transporter (DAT) in response to the substrates, amphetamine, and dopamine,[27] and to study the purinergic-signaling cascade by directly visualizing ATP-loaded vesicles and their fusion to the plasma membrane.[28] Also, cancer screening agents such as QDs doped with ORMOSIL, which were stained on the cell membrane, were tested as optical probes.[29]

Another group used TIRFM to propose a fibroblast reorientation scheme.[30] They mapped the spatio-temporal dynamics of cell protrusion/retraction and Pl3K signaling, which lead them to determine that randomly migrating fibroblasts reorient polarity through Pl3K-dependent branching and pivoting of protrusions.

TIRFM has also recently been used to study the dynamic coordinated cytoskeletal rearrangements in drosophila by visualizing the cortical events with better spatial and temporal resolution,[31] and to study Eg5, a member of the kinesin-5 family, and its spatial-temporal distribution in mitosis.[32] The TIRFM results demonstrated that Eg5 dynamics within the mammalian spindle are region-specific, that the motor reorganizes at the different stages of mitosis, and that its dynamic reorganization is mediated by dynein and TPX2.[32]

Förster resonance energy transfer (FRET) benefits from the background reduction associated with TIRFM. TIRFM has been employed to visualize the real-time conformational changes in the actin transformation and correlate these changes to the presence of myosin.[33] FRET has been used on the plasma membrane to study SNARE interactions in living cells[34] and has been extended to the investigations of apoptosis by monitoring caspase activities.[35] The same authors have designed a FRET-based TIRF reader taking advantage of multiple TIR reflections for detection of apoptosis, drug screening, or in vitro diagnosis.[36]

Controlling the polarization of the incident illumination in TIRFM can divulge information about the fluorescent probe orientation and concentration. Two polarizations are commonly utilized: s-pol (perpendicular to the plane of incidence and parallel to the TIR surface) and p-pol (parallel to the plane of incidence and perpendicular to the TIR surface). Oriented fluorescent probes will fluoresce accordingly to the incident polarization. The simple ratio of p-pol/s-pol (P/S) images will mark deviations from sample uniformity, while P+2S is proportional to the effective concentration. Recently, the topological changes of chromaffin cells were monitored through the process of exocytosis.[37] As exocytosis occurs, the orientation of the labels attached to the membrane changes before resuming their original conformation.

Plant Cell Imaging. While TIRFM has a long-standing history in imaging and molecular tracking in animal cells, historically, applications have been limited involving plant cells. The single-most restricting factor to plant cell imaging is the thickness of the cell wall, which varies widely between species but is typically several hundreds of nanometers thick (>250 nm). Unsurprisingly, this has limited the use of TIRFM to in vitro investigations of actin cytoskeleton,[38, 39] or to investigations near new growth where the cell wall is still relatively thin.[40] VAEM has been demonstrated to circumvent the challenges posted by cell walls in plant cell imaging.[11] While not truly TIRFM, the thin stepwise sample penetration keeps the advantages of optical sectioning, low background, and reduced photobleaching of the sample.

In 2011, two groups both found they could produce TIR at the inner boundary of the cell wall.[41, 42] As previously mentioned, TIR occurs when light passing through one medium reaches the interface of second medium of a lower *n*. A typical cell wall has *n* between 1.42 and 1.48 bordering the cytosol, which has *n* of 1.38. Using variable angle systems with fine angle control allowed the researchers to move between glass slide TIRFM, VAEM, and cell wall TIRFM. Both groups were able to produce high-quality images of cytoskeleton and organelle markers. Wan et

al. were able to track a membrane-associated receptor and GFP labeled clathrin light chains with both VAEM and TIRFM observing different intensity traits.[41] Vizcay-Barrena et al. concluded single-molecule analysis of EGFP is possible within root cells using TIRFM (Figure 2).[42] Observing these events under TIRFM laid to rest the commonly held belief that TIRFM has little value in plant cell imaging.

Total Internal Reflection Scattering Microscopy

Total internal reflection scattering (TIRS) microscopy is a technique where, similar to TIRFM, the illumination light undergoes total internal reflection at the sample-substrate interface. Instead of a fluorescent signal, the collected light is scattered from the sample. Originating as total internal reflection microscopy (TIRM), the technique was used to inspect surfaces for damage induced by lasers.[43] Since then TIRS microscopy has been used to measure colloidal and hydronamic forces,[44-46] and eventually became used to monitor the cellular uptake of sub-micron non-fluorescent particles.[47]

Recently TIRS microscopy has been applied to single metallic nanoparticle orientation determination.[48, 49] Metallic nanoparticles placed near a dielectric surface with high permittivity and illuminated with a frequency of light that aligns with the surface plasmon resonance of the particle will undergo coupling of the collective plasmon modes with the induced image charge.[50-52] This effect is reliant on the polarization of the incident light. If the light is s-pol, the induced image charge will negate the plasmon resonance of the particle, and no scattering enhancement will be seen. If the light is p-pol, the image charge will couple and enhance the plasmon resonance producing an image that is “doughnut” in shape.

An anisotropic particle that has an out-of-plane (tilt) angle with respect to the substrate plane will induce an image charge based on the 3D orientation of the particle.[48] A gold nanorod (AuNR) lying flat upon the surface will produce a “doughnut” shape with an intensity profile that alludes the direction of the long axis. If the AuNR is tilted the image produced has a “pacman” shape. The image profile will eventually become Gaussian in shape when the AuNR is oriented perpendicular to the surface. Through simulations of these interactions it is possible to match patterns to estimate the 3D orientation of the dielectric anisotropic nanoparticle.

Differential Interference Contrast Microscopy

Differential interference contrast (DIC) microscopy is a technique typically used to enhance the contrast of unstained samples. Also known as Nomarski interference contrast or Nomarski microscopy, it was invented by Georges Nomarski in 1955. Compared to bright field microscopy, the optical components are decisively more complex. A schematic of the light path can be found in Figure 3.

A polarizer is first used to produce a polarized illumination source, which is then split into two orthogonally polarized wavefronts by a Normaski prism. These wavefronts are separated by what is called the sheer distance. This causes the two wavefronts to pass through a slightly different portion of the sample, thus being affected by the sample to different extents resulting in a phase delay. A second Nomarski prism is then used to recombine the orthogonal wavefronts. The phase delay will cause either constructive or destructive interference resulting in an image consisting of a gray background with signal either being lighter or darker than the background.

DIC microscopy produces characteristic images that appear as though they are being illuminated by a point source. Features within the image have a bright and dark side and appear to be three-dimensional. The high NA condenser and objectives that are used enables the DIC to image a very thin section of the sample due to the depth of focus. This leads to an improved image quality due to little out of focus light being captured.[53]

Since DIC microscopy does not require the staining of the specimens, it is commonly used in cell imaging. The technique can resolve features as small as the microtubules that make up the cytoskeleton of the cell (25 nm in diameter)[54-56] and has been used to monitor cell functions such as the motility of bacterial flagella,[57] and the assembly of sickle cell hemoglobin filaments[58]. DIC microscopy has even been used to differentiate between either gold or silver metallic nanoparticles as small as 10 nm, and has been used to follow the real-time particle endocytosis in HeLa cells.[59] Recently, the shadow-effect patters intrinsic to DIC microscopy have been utilized to resolve the orientation information of anisotropic nanoparticles in controlled environments[60] and within cells.[61]

Dissertation Focus

This dissertation focuses mainly on the application and advancement of TIRF, TIRS, and DIC microscopy. TIRF and TIRS microscopy were used for investigating single-particle motion and rotation on biologically relevant systems, while a homebuilt Nomarski DIC microscope was built in reflection-mode to study nanoparticles and nanostructures on reflective surfaces. The work was carried out in three main directions:

(1) Implementation of "non-blinking" CdSe/CdS nanocrystal quantum dots to investigate biological systems. Semiconductor quantum dots (QDs) are fluorescent particles that have intrinsic properties that hold advantage compared to organic fluorophores such as broad absorption and narrow size-tunable emission spectra[62, 63] and increased photostability.[64] Despite all these advantages, QDs have the intrinsic trait of fluorescence intermittency commonly referred to as "blinking". This is a significant drawback in single-particle tracking (SPT) experiments when trying to continuously determine their absolute positions. While some methods have been established to reduce fluorescence intermittency by modifying the surrounding environment,[65, 66] a new type of core/shell QD has been introduced that has drastically improved blinking statistics.[67-69]

The new QDs, referred to as "non-blinking" QDs (NBQDs), have enough fluorescent stability to be implemented as single-particle probes for investigating the movement of molecular motors and the nanometer vertical displacements they can induce by scanning-angle TIRF microscopy. Varying the angle of incident illumination directly affects the depth of the EF and therefore the intensity of the particle's fluorescence.[7] The NBQDs were attached to stationary microtubules while the fluorescence intensity was recorded as a function of incident angle. The curve of the plot revealed the vertical position of the particle. Precise control of the EF can allow for the tracking of nanoscale vertical displacement of NBQDs as non-13-protofilament microtubules propelled by the motor protein kinesin rotated across the substrate surface.

In a separate experiment, the surface loading of NBQDs with a cholesterol derivative was controlled with respect to complete PEG coating. Cholestanone is known to incorporate itself with phospholipid membranes. Observations showed that movement of the particle across the lipid bilayer surface is directly related to the amount of particle surface occupied by cholestanone. Calculations of the number of cholestanone molecules infused with the surface creating a drag force opposite particle diffusion matched well with observations.

(2) Advancing TIRS microscopy for the determination of the 3D orientation for dynamic and static nanoparticle. Techniques under dark field (DF) microscopy have used AuNRs as orientation probes as these particles preferentially scatter light along their longitudinal axis.[70-72] Though DF is a convenient technique, it has only been able to resolve orientation in two dimensions. Intrinsic to the nanoparticle is the surface plasmon resonance (SPR), which is the oscillation of free electrons in the metal at a certain frequency. Because of the rod shape of AuNRs there is both a short and long axis SPR. When a polarized light source with the same frequency of the SPR illuminates the particles, an enhancement of the SPR will result in an increased scattering signal.

A dual-color TIRS microscope was constructed that can observe both SPR enhancements simultaneously using a dual-view system. By manipulating the polarization orientation of the incoming laser lines, in-plane and out-of-plane (tilt) orientation information of the AuNR could be resolved. The scattering profile produces a Gaussian point spread function profile that can be super-localized. Combining the orientation and location information, a study of particle-lipid membrane interaction was performed.

In a separate experiment, the 3D orientation of AuNRs was determined for stationary particles. By rotating the polarization direction of incoming linearly polarized light, different extents of s-pol and p-pol are produced in the evanescent field under TIR. The scattering intensity maximum for a particular AuNR is dependent upon the 3D orientation of the particle. By matching the scattering intensity profile of a particle with its in-plane orientation, the polar angle of the particle can be accurately determined.

(3) Developing a reflected-mode Nomarski differential interference contrast microscope for the study of nanoparticle and nanostructures. Traditionally, DIC microscopy has been used to study systems on transparent substrates using a second Nomarski prism after the sample to recombine the sheared light. In reflection mode, there is need for only one Nomarski prism placed in the back focal plane of the condenser as the condenser also acts as the objective. The single Nomarski prism both shears and recombines the light. Reflected DIC was developed in 1979 primarily for use in surface topography studies,[73] though it is currently a convenient method to inspects chips based on silica wafers.

A reflected-mode Nomarski DIC microscope with a laser illumination source was constructed to image nanoparticles and nanostructures developed on reflective surfaces. The

setup can distinguish 3D orientations of gold nanorods under the axial SPR excitation mode. The study was extended into imaging nanostructures created through lithography on silica wafers.

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