6.0 MOLECULAR AND CELLULAR IMAGING

Abstract

This project will focus on identifying and characterizing the multiprotein complexes that execute cellular functions and govern cell form. Much of the proposal Core and several of the subtasks are focused on the discovery of these protein machines. This Molecular and Cellular Imaging subproject has a two-fold mission: validation of the discovery findings and characterization of the fundamental functionality of machines. The validation work will address uncertainties related to the process of extraction, manipulation and analysis of complexes from cells, and whether the results faithfully reflect the existence and composition of complexes in living cells. The characterization work is focused on providing a molecular scale understanding of the machines and an in vivo understanding of their existence and location within cells and on cell surfaces. New tools will be developed that are based upon hybrid and multifunctional imaging methods, combining AFM, single molecule and non-linear optical approaches. New nano-scale probes will also be developed in order to better understand the expected relationship between cell function (where, when and to what extent particular machines exist) and cell environment. In this project we describe a series of technical approaches to meeting these needs. These tasks will be pursued as the products and information from the core and other research task mature. Initial emphases will be placed on the first two of the following specific aims:

Specific Aim #1: Develop methods for analysis of individual protein complexes to discover the relationship between the average complex composition to the actual composition of each complex contributing to that average.

Specific Aim #2: Develop multimodality AFM and optical imaging approaches to the study of cell surfaces and the extracellular environment to determine what complexes are active at or near the cell surface, what is their distribution and how are these factors influences by the nature of the extracellular environment.

Specific Aim #3: Create new tools for the characterization of the complexes in terms of their topology, dynamics and binding forces in order to relate their existence to function.

Specific Aim #4: Extend the time resolution of optical probes for protein co-localization measurements for application to bacteria, enabling direct, in vivo measurements of protein complex existence and the related formation kinetics and dynamics.

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6.2 Background and Significance

The primary requirement for imaging as part of Goal 1 research is validation of the existence and function of protein complexes in living cells. Do the protein complexes discovered through component analyses exist as such in living cells, e.g. do the methods used to extract complexes retrieve intact functional units? Also, do the complexes conduct the biological function expected from knowledge of their corresponding genes, can one complex provide more than one function itself or by working in concert with other proteins? Another important impact will be the ability to label and track important complexes in a variety of cells and under a variety of conditions. A molecular scale description of protein interactions, in real time, will reveal metabolic relationships. This will complement information obtained by mass spectrometry and other methods, allowing molecular level biological information to be obtained as a function of conditions. This would also reduce the requirement for analyses of the proteome under all conditions and as time proceeds during the evolution of a microbial colony. Finally, imaging methods will be valuable in characterizing cultures, sorting cells and performing similar functions as part of the sample preparation process. The more information known about the state of the biological system at the time of a MS analysis for example, the more certainty can be placed upon interpretation of the resulting data.

Microbes present particular challenges due to their small size, minimal organizational substructure and impervious cell walls (compared to eukaryotes). New and innovative methods will be required to provide the level of detail required to validate complexes in living cells. Our approach will be to develop novel intra- and extra-cellular probes of cellular function, striving for temporal and spatial description of the molecular components of the cell that will enable an unprecedented description of cell function.

Success in this endeavor will require close coupling to other complementary information and techniques. Structural biology, synthetic chemistry and pathway identification will be needed to complement advanced approaches to imaging. For example, knowledge of complex secondary structure, either directly through structural biology (SAXS, SANS) or indirectly through mass spectrometric-based crosslinking and isotope exchange studies, is vital for directed attachment of extrinsic probes (e.g. fluorescent) at sites that do no perturb protein function. Synthetic techniques will be needed for modifying proteins or imaging probes. Metabolic pathways will be needed as targets for validation and for deducing the potentially multiple functions of a protein complex. Most significantly, the imaging tools proposed here will require a tight association with the core efforts of the Genomes to Life Center for Molecular and Cellular Systems and supporting resources. The protein complexes defined by the Center High Throughput Molecular Characterization subproject will provide initial targets for in vivo characterization. Close integration will be necessary to learn of the existence and nature of protein complexes that are thought to have important biological functions. The research conducted as part of the High Throughput Complex Isolation subproject will be vital for production of complexes, labeled derivatives and molecular recognition agents that will be used in imaging studies. Finally, the Bioinformatics and Computing thrust will be essential for prediction of complex formation and for assistance in handling and analyzing the vast amounts of data that result from image collection. An iterative relationship with computational biology research will provide for synergistic feedback on the functionality (within the broader context of cellular and multicellular systems) of specific molecular machines.

Currently, several biophysical techniques are used to assess cellular function. Fluorescence microscopy techniques prevail for the in situ localization and identification of molecular species within cells. However, the resolving power provided by optical techniques constrains the spatial resolution of labeled species. Fluorescence resolution elements are typically too large to provide the detailed positional information that is sought, resulting in only a course-grained image of the cell. Several approaches can be considered to address this limitation, including near field optics, non-linear optics and multiphoton excitation. These techniques can reduce the spatial dependency imposed by the diffraction limit enabling spatial resolution down to ~50 nm.

An additional limitation of fluorescence imaging is that the diffraction limit poses serious limitations on information content. The excitation-volume ellipsoid has a volume that can be a significant fraction (10 – 30%) of the cell volume. One potential solution to this problem is to find a new class of fluorescent species that respond spectrally in well-defined ways to small changes in local environments. This adds a spectral dimension to the

problem that could, in principle, be used to determine local changes in the cell's microenvironment. This could complement image analysis with information on local temperature, ion concentration and pH.

Other approaches to imaging will also be necessary for describing protein complex function and location. The dimensional scale of biological systems is remarkable and presents a significant obstacle for analytical measurements. Typical bacterial cells are only a few microns in diameter resulting in fluid volumes of a femto-liter. The containing membrane is a few nanometers thick, and the molecular complexes we seek to define can extend from a few nanometers to tens of nanometers in size. To address this sub-optical length scale and required sensitivity, relatively unconventional approaches to cell imaging will be needed. The emerging science of nanotechnology is poised to meet many of these requirements. Nanotechnology addresses the science occurring at length scales of less than 100 nanometers. A cornerstone characterization tool of nanotechnology is the scanning probe microscope (SPM). Versions of the SPM can provide molecular level detail of biological systems in their natural environment. Sampling over nanoscale dimensions enables assessment of single complexes, leading to their identification and physical characterization. Such molecular level characterization can overcome the limitations of conventional analyses that often average and overlook low population or transient complexes. The combination of SPM with other imaging modalities presents a powerful technique for molecular and cellular imaging.

Other aspects of nanotechnology focus on materials synthesis. A wealth of materials are becoming available that may enable interpretation and validation of molecular complexes and cell function. An example is shown at right (**Fig. 6.2.1)** where nanoscale carbon fibers are interacting with a cell. Intra- and extra-cellular probes are readily envisioned. The small size of this new generation of synthetic materials can match effectively the scale of cellular detail. These materials offer new approaches for assessing protein complexes and for potentially interfacing to cellular function. Nanoscale materials also offer superior physical properties over conventional materials (e.g. strength, thermal and electrical conductivity, optical properties). These properties can lead to the realization of optical, magnetic and electronic probes that directly interface, or assess, molecular complexes while maintaining the capability for high spatial and temporal resolution. The emerging resources in nanotechnology (e.g. ORNL's Center for Nanophase Materials Sciences) will provide a valuable resource for meeting future imaging requirements.

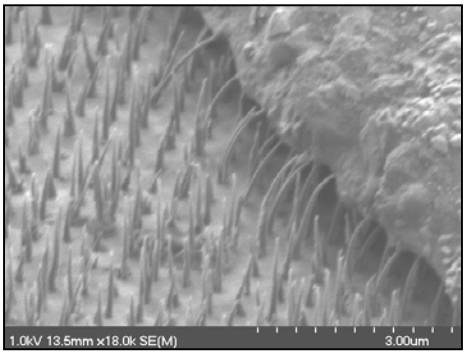


Fig. 6.2.1. An array of vertically aligned carbon nanofibers (~50 nm diameter, 2 μm tall) interacting with a bacterial biofilm.

Our initial objectives will be directed towards validating protein complexes investigated by the Center. Initial efforts will focus on validating and characterizing the intracellular hydrogenase complex and the membrane bound nitrogenase complex in *Rhodopseudomonas palustris*. Optical and scanning probe techniques will be used. Concurrently, efforts will focus on developing advanced optical tools, advanced iterations of scanning probe microscopes and novel nanoscale probes for characterizing protein complexes. These tools will be applied to complexes that occur intracellularly, within the cell membrane and within simpler systems that attempt to mimic the complex's natural environment. In all cases, we will strive for real-time, molecular level analyses. Our goal will be to provide imaging tools that allow for the routine characterization of cellular events at the molecular scale.

As outlined in the Overview to this proposal, the projects described are the initial research projects being proposed to support the Center for Molecular and Cellular Systems. These projects will be reviewed annually as outlined in the Management Overview for progress and continued relevance to the Center. Normally no project will extend beyond two years. It should be noted that techniques developed as part of this project will be incorporated into Center sites at both ORNL and PNNL. The activities of this subproject will necessarily be smaller at the outset of the project due to priority given to establishing methods for high throughput isolation and identification of protein complexes. Also, the research activities in this subproject will benefit from data being generated in the core. The research described in this subproject is central to relating the relationship of the protein

complexes to biological function. Thus, it is expected that these research activities will grow during the course of this project.

6.3 Preliminary Studies

The status of current methods for observing the function of individual (or small groups of) molecular complexes in real-time, in live cells and simulated situations (with a high degree of environmental control) through a variety of approaches is described in the following. Various techniques are in place and under development at ORNL and PNNL. These techniques include variations on scanning probe microscopy, optical spectroscopy as well as unique exploitations of nanoscale materials. All these techniques have the common capability of high sensitivity, some extending down single molecule sensitivity. They differ by the type of information that is obtained and the method for doing so. These techniques form the basis of our research thrusts discussed in Section 6.4.

6.3.1 Nano-Scale Probe Techniques

Scanning probe techniques comprise a variety of imaging techniques that have in common the raster scanning of a nanoscale probe. This technique provided a fresh approach to imaging biological macromolecules and cell surfaces and started with the invention of the atomic force microscope (AFM), by Binnig, Quate, and Gerber [Binnig, 1986] in 1986. Unlike the conventional high-resolution electron microscope, which must operate in a vacuum, the AFM is a surface sensitive instrument capable of imaging biological samples, at nanometer resolution, in native environments. The unique characteristics of imaging with a position sensitive probe allow the AFM to image biological samples in liquids and without chemical treatment. This permits imaging of functional biomolecules and living organisms, including both bacteria [Sukolov, 1996; Ong, 1999; Bolshakova, 2001] and cultured cells [Henderson, 1992; Hoh, 1994; LeGrimellec, 1994, 1997; Rotsch, 2000], in medium where both composition and temperature duplicate the natural physiological environment. The extreme sensitivity of the micro-cantilever to contact forces [Hoh, 1992] used to image soft biomolecules on surfaces, has also been exploited to identify and measure force interactions in the pico Newton (pN) range, within [Rief, 1997, 1999; Muller, 1999; Oesterhelt, 2000; Oberhauser, 1998; Zhang, 2000] and between [Boland, 1995; Han, 1995; Lee, 1994a, 1994b; Ludwig, 1994; Florin, 1994] individual biomolecules. By attaching specific antibodies to cantilevers simultaneous imaging of target antigens and identification of antigen-antibody interactions was demonstrated [Haselgrubler, 1995; Hinterdorfer, 1996; Baumgartner, 2000]. Nano-scale probes are also effective means for characterization the metabolic state of bacterial cells and their environments as will be discussed below.

6.3.1.1 Imaging Protein-DNA Complexes The AFM is capable of resolving individual DNA molecules, with bound proteins, under optimal in vitro binding conditions. Some cellular processes such as RNA polymerase movement on individual DNA molecules were imaged by AFM [Thomson, 1996]. Simultaneously, AFM is capable of determining protein size, and (quantum) numbers of proteins in a complex, for example, AFM imaging resolved protein multiplexes that exist in vivo but are irresolvable by other techniques [Wyman, 1997]. Thus AFM can resolve spatial and temporal aspects of functional protein complexes.

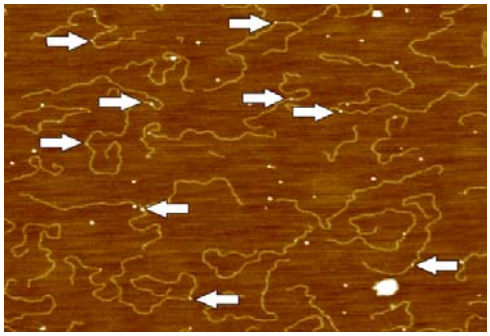


Fig. 6.3.1. AFM image of MutS protein bound to purified heterologous DNA derived by heteroduplexing plasmids with a single base change. The resulting DNA has a single base (G→T or C→A) mismatch. Arrows denote protein binding of MutS to the predicted position of the lesion site.

Prior work in our laboratory has demonstrated the ability to image biomolecular complexes. For example, we pioneered the use of restriction enzymes to map sequence locations on large DNA molecules by AFM imaging [Allison, 1996, 1997]. The binding sites of cleavage deficient EcoRI molecules could be precisely measured to within a few nanometers on cosmid and BAC sized DNA clones. Other protein-DNA complexes, useful in detecting sequence polymorphisms or understanding DNA repair mechanisms, have also been studied. Shown in **Fig. 6.3.1** are images of the repair enzyme MutS [Smith, 1996; Wagner, 1995]

bound to single base pair mismatches on plasmid DNA. These studies directly relate to understanding the DNA repair protein components of cells at the single molecule level. Additional manipulations of this system could lead to an in vitro system capable of modeling DNA repair, and a greater understanding of how transcription-coupled DNA repair is associated with cell phenotypes. Another protein-DNA complex is shown in **Fig. 6.3.2**. Here, a gene expression regulatory structure, with multiple protein-protein and protein-DNA interactions, is shown bound to a promoter sequence. The protein complex was prepared using whole cell extracts. Essential for these experiments are methods for reducing non-specific binding. We have developed such methods using molecular exclusion to separate the unbound proteins from the proteins bound to the DNA molecule [Hoyt, 2000].

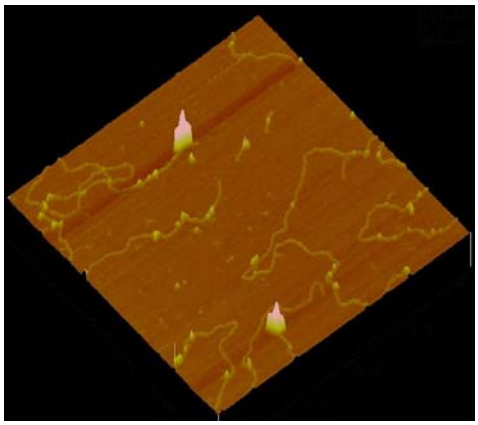


Fig. 6.3.2. Imaging complex protein mixtures such as HeLa cell nuclear extracts gives us an indication of the position of forming transcription complexes, and could result in the ability to map and quantify gene positions in unknown genomes, or annotate sequenced genomes. Shown is a Linearized pAD-Beta Galactosidase plasmid vector treated with HeLa cell extracts. A remarkable tripartite structure has fomed on the promoter region of some of the molecules.

6.3.1.2 Imaging cells

The impressive atomic level resolution provided by scanning probe techniques can also be performed on larger structures such as whole cells. Shown in **Fig. 6.3.3** are *Escherichia coli* K12 cells imaged under two different conditions, dried or under liquid. The cells were first grown on LB agar overnight, scraped off the agar surface and suspend in 100 mM sodium chloride. For imaging in air a droplet of suspended cells was placed on a freshly cleaved mica surface and allowed to dry prior to scanning. For imaging in liquid a droplet of suspended cells was placed on a mica surface, pretreated with polylysine and flushed with 100 mM sodium chloride. These experiments indicate the exciting possibilities of imaging protein complexes in live cells, in real time.

6.3.1.3 Nanoscale Electrochemical Probes for Intra- and Extracellular Analyses

Microelectrophysiological electrochemical measurements, i.e., electroanalytical detection techniques, performed at ultramicroelectrodes (<50 µm) are emerging as a promising avenue for the interrogation and monitoring of chemical dynamics at the single cell level [Huang, 1995a]. The promise of these electrodes lies in their fast response times (ms time scale), high mass sensitivity (zeptomole, i.e. 100-1000 molecules), small size, large linear dynamic range (up to 4 orders of magnitude) and selectivity, and the fact that molecules of interest can be followed without the need for derivitization as is necessary in fluorescence microscopy. In addition to a wide array of electroactive compounds (e.g., neurotransmitters, peroxides and peroxidation products, oxygen) compounds such as nitric oxide [Malinski, 1992, 1993; Brovkovych, 1999], glucose [Abe, 1991; Netchiporouk, 1996], and insulin [Kennedy, 1993; Huang, 1995b] have been monitored in or near single cells with no measurable interference from other easily oxidizable compounds [Huang, 1995a].

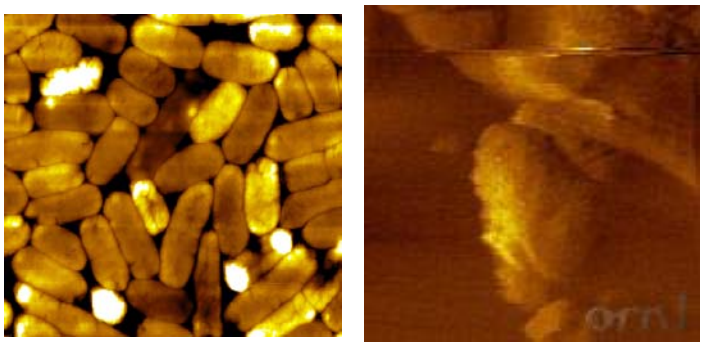


Fig. 6.3.3. Images of E. coli imaged in air (left) or under liquid (right). The image in air was collected in contact mode while imaging in liquid was by MAC-mode using a Molecular Imaging PicoSPM AFM.

Our group has developed a unique new platform for cellular electrophysiological measurements

that exploits arrays of vertically-aligned carbon nanofibers as multipoint, individually-addressable, nanoscale electrochemical probes [Guillorn, 2002]. An example of an array of fibers, interacting with a biofilm, was shown previously in **Fig. 6.2.1**. These devices feature active probing tips with diameters of ~30 nm (or smaller), providing a molecular probe at size scales far below the diffraction limits of optical/fluorescent microscopy. Nanofiber-based probe arrays provide a powerful platform for a diverse array of electrochemical studies within and around individual cells and cell groups. Chemically derivitized sensors may also be generated to enable probing for non-electrochemically active species – as affinity based probes for specific proteins or nucleic acids. Affinity sensing is achieved by physically binding receptor sites upon the carbon nanofiber scaffold. A variety of chemical derivatization approaches have been developed. These structure can be electrically addressable as shown in **Fig. 6.3.4**. Analytes can be detected via the impedence changes that occur to the electrochemical boundary layer due to the presence complexing species. Nanofiber based hybridization probes feature geometries that should enable directly locating probes and probe arrays into cells for the specific detection of proteins, measurement of particular metabolites or oxidoreductase activity.

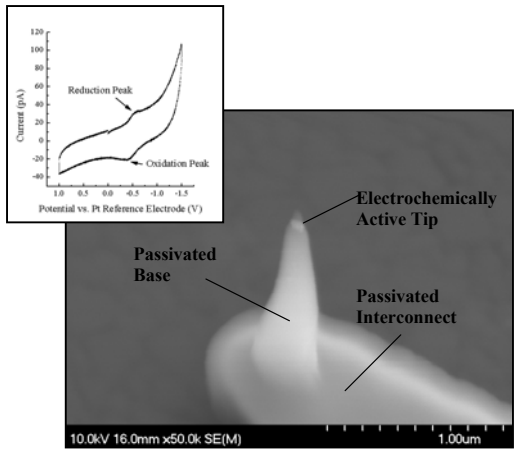


Fig. 6.3.4. An individually addressable carbon nanofiber cellular probe, featuring a 30 nm diameter tip upon a 2 µm vertical sheath. The electrochemical response of these probes (inset) enables analyte probing at resolutions exceeding the diffraction limit of optical and fluorescent microscopy.

6.3.2 Optical Probing Techniques

6.3.2.1 Intracellular Imaging Methods

We are developing new microscopy methods that provide information and capabilities unavailable from traditional fluorescence imaging. Existing methods for labeling specific cellular components (for example, particular proteins) require antibody labeling and are not easily applied to intracellular imaging of live bacterial cells. Some other extrinsic labels are useful for live cell imaging, but there are phototoxicity and photobleaching problems restricting their usefulness, which are also problems with GFP labels. Raman imaging, which has molecular specificity, has sensitivity problems, which are substantially improved with a CARS (Coherent Anti-Stokes Raman Scattering) imaging approach. This is a form of multiphoton microscopy that detects molecules based upon their intrinsic vibrational spectrum and hence is not constrained by the labeling, photobleaching, and phototoxicity of fluorescent microscopy methods. Abundant molecular species may be mapped in three dimensions, as in two-photon fluorescence microscopy. We introduced the high-resolution collinear method [Zumbusch, 1999], and have developed the technology to permit accessing a wide range of frequencies [Holtom, 2001b]. We are also investigating the use of deuterated probe molecules, which have strong Raman features in a spectrally uncongested region, improving the contrast and sensitivity for studies in lipid transport [Holtom, 2001a]. When intracellular fluorescent probes are available, multi-mode imaging using two-photon fluorescence and CARS simultaneously is both reasonable and useful for developing new imaging strategies [Holtom, 2001b].

Our ongoing development efforts are taking two complimentary directions. First, we are adapting available laser technology to provide convenient means to tune the laser frequency and control the laser linewidth [Holtom, 2001b]. Secondly, we are interested in understanding the detailed physics of signal production, particularly the contributions to the detected signal from non-resonant sources, or broad, undesired components such as water bands. Significant advances in CARS microscopy have been made in other laboratories to improve contrast and to differentiate the signal from small features present in cells. New methods include using epi-collection of the signal [Cheng, 2001a], using polarization to remove electronic contributions [Cheng, 2001b], and taking advantage of the persistence of the vibrational coherence [Volkmer, 2002]. We have the capability for epi-CARS and polarization-CARS at present, and are developing time-domain CARS which is probably the most useful of

the new methods. Unfortunately all of these methods to improve contrast do so at the expense of most of the signal. We are exploring ways to tailor the laser properties (spectral width, frequency, bandwidth, repetition rate) to fully optimize the image contrast with as small a sacrifice in signal level as possible. For example, we wish to improve our imaging time from minutes per two-dimensional image slice to seconds, using higher repetition rate laser technology.

6.3.2.2 Molecular Dynamics Measurements for Isolated Complexes

Single molecule methods have recently been developed that enable direct measurements of reaction dynamics (for each step in a complex reaction) without averaging over the numerous similar (but importantly different) reactive species in an ensemble sample. Single-molecule enzymatic reactions have been studied under physiological conditions in recent years [Polakowski, 2000; Xue, 1995; Lu, 1998; Weiss, 1999; Edman, 2000; Zhuang, 2000]. Single-molecule assays have revealed static [Xue, 1995; Lu, 1998; Edman, 2000] and dynamic disorder [Lu, 1998; Edman, 2000] in the rates and dynamics of single-molecule enzymatic reactions [Polakowski, 2000; Xue, 1995], co-enzyme redox state turnovers in real time [Lu, 1998], and enzymatic reaction product formation in real time [Edman, 2000]. We have recently applied this approach to better understand the hydroslysis of bacterial cell walls by the T4 lysozyme enzyme. The enzyme was labeled with a donor/acceptor dye pair at specific external sites so as not to modify its function. Enzymatic reaction turnovers are observed during hydrolysis of bacterial cell walls. The data in the three panels of **Fig. 6.3.5** were recorded at 0.65 ms per channel for the same reaction conditions. The upper panel shows an extended portion of middle-panel trajectory of the donor fluorescence of a donor-acceptor labeled single-T4 lysozyme. Intensity wiggles in the trajectory are evident. The lower panel shows a portion of a trajectory recorded from a donor-only-labeled protein. The fluorescence intensity distributions derived from the two trajectories are shown in the insets of the middle and lower panels. The solid lines are fits using single and bimodal Gaussian functions, respectively. The formation of ES and ES* involves significant domain breathing motions along the α-helix "hinge" causing the large amplitude wiggles and the bimodal amplitude distribution. Statistical analysis of the data provided detailed kinetics for each step of the enzymatic reaction. [Lu, 1998; Xie, 1999; Chen, 2002]

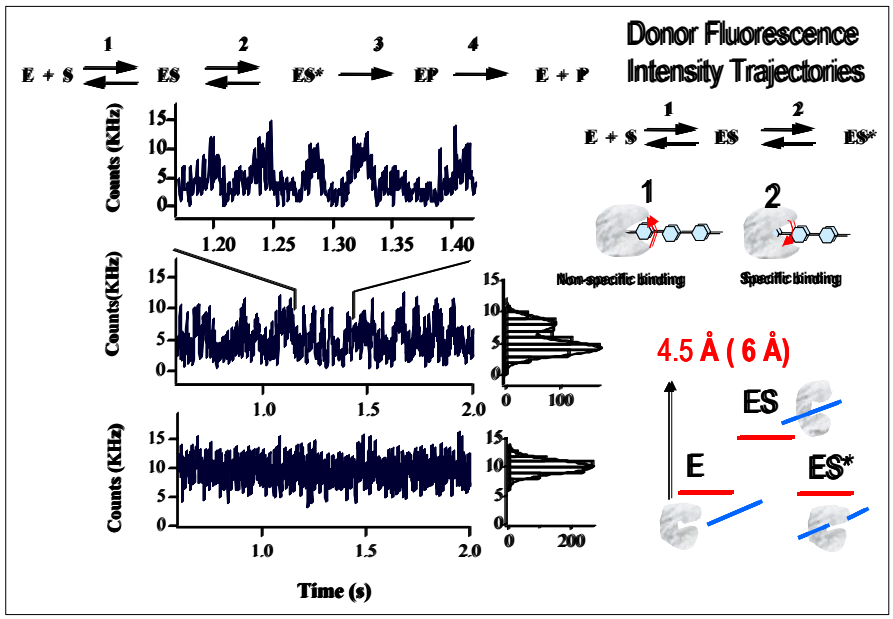


Figure 6.3.5: Real-time observation of single-T4 lysozyme conformational motions using spFRET where donor and acceptor dye molecules have been substituted on the extremities of the enzyme.

Such results benefit from advanced computational methods for modeling the observed results and predicting the effects of structural changes using an approach that merges the computational and single molecule tools to greatly enhance the value of reaction simulations. The conformation trajectories of T4 lysozyme in solution at room temperature can be calculated by MD simulation [Chen, 2002]. The microscopic conformational change mean drifting velocity <v>, diffusion coefficient D, friction coefficient ξ, and the energy spent on the friction along the reaction coordinate E_ξ can be obtained by this combined approach of MD simulation, theoretical modeling, and single molecule experimental trajectory [Chen, 2002]. Based on the single-molecule recording mode-specific conformational change dynamics and MD simulation, we are now able to reveal the fine details of the enzymatic reaction active complex formation dynamics and mechanism.

6.3.2.3 Advanced Optical Probes

Quantum dots, fluorescent dyes, and dye-doped polymer spheres have all received some attention for bioconjugate applications where time and/or spatially resolved fluorescence from the conjugate is used to provide "when and where" information on the transport of the biomolecule in question.

We have recently shown that single rare-earth ions, such as europium and terbium, doped in metal oxide host crystals can be isolated and imaged using far-field high numerical aperture microscopy techniques commonly used in biological contexts [Barnes, 2000]. These species represent an interesting new class of nanoscale materials where the optical properties are defined by the dopant ion and are not strongly sensitive to the choice of host material. Our research goal is to explore ways in which these new materials can be exploited for intracellular monitoring applications.

In exploratory experiments, dry Eu^{3+} (or Tb^{3+}):Y_2O_3 nanoparticles ranging in size from 2 – 15 nm (characterized by *in situ* AFM) on a quartz slide were continuously illuminated with the 514.5 nm line from an Argon ion laser in an epi-illumination configuration. [Barnes, 2000] Luminescence from Eu^{3+} doped nanocrystals appeared as diffraction-limited spots with intensities ranging from 200 – 1000 counts above background per frame (200 ms exposure). **Fig. 6.3.6** shows spectra from two different Eu^{3+}:Y_2O_3 nanoparticles (12 and 5 nm diam.) illustrating the well defined spectral nature of these species. One of the most surprising and interesting features of this system is that the blinking is *not* binary: When the luminescence turns "on", we observed three different discrete intensity levels, or bright states. We also observe "random-access" behavior in switching between different bright states; any one particular bright state can be accessed from any other. To our knowledge this multiple bright state behavior is unique to rare-earth doped nanocrystals and we believe its origin lies in local symmetry fluctuations in the crystal.

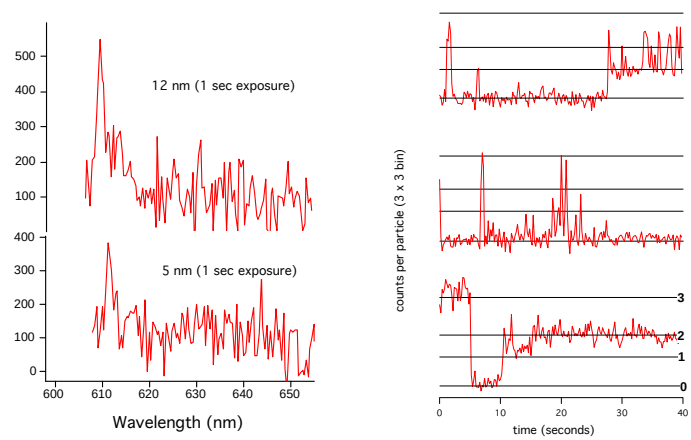


Fig. 6.3.6. (Left) Luminescence spectra from individual europium doped nanocrystals illustrating the well defined spectrala signature of Eu3+. (Right) Integrated photon counts as a function of time from single Eu3+ ions in doped nanocrystals illustrating multiple bright state behavior.

In related work, we have developed techniques for precise size-correlated optical imaging and spectroscopy of nanoscale species. Integrated on a research-grade microscope platform, we have combined a large area AFM scanner and high-numerical aperture far-field imaging or spectroscopy that can simultaneously monitor hundreds of luminescent entities (single-molecules, quantum dots, etc.) within the field of view with a time resolution of a few microseconds. **Fig. 6.3.7** shows an example of size-

correlated luminescence measurements made on a polydisperse Eu^{3+}:Y_2O_3 nanoparticle sample. The red contours indicate a 3-nm surface height and the yellow indicates 5 nm (greater). We will explore ways in which this instrumentation can be modified to perform in situ analysis of proteins or other similar species alone or in combination with other imaging techniques.

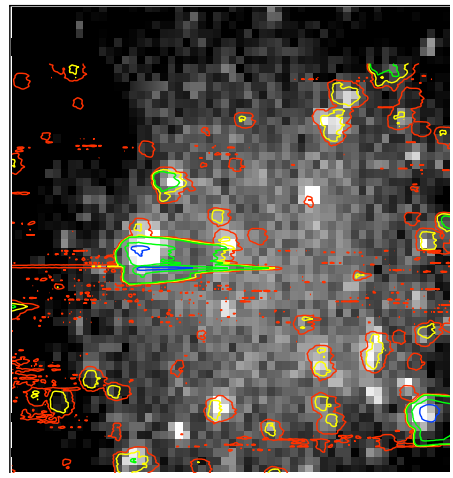
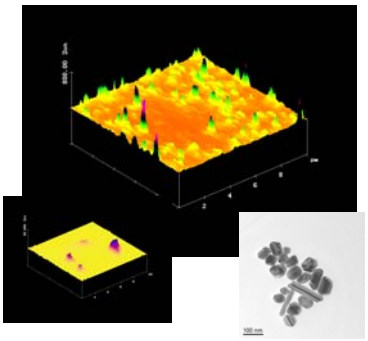


Fig. 6.3.7. Example of size-correlated fluorescence imaging on a polydisperse europium-doped nanoparticle sample. The X and Y frame dimensions are 20 um.

Another combined microscope that uses the Optical/AFM approach and a high-sensitivity far-field microscope has also been developed to provide unperturbed measurements of biological dynamics at sites that have been characterized by AFM imaging simultaneously and/or consecutively. Two microscopes have been combined in an over/under arrangement, with both imaging devices set to observe the same point in space. These new approaches have been built upon techniques and results from previous fundamental sciences research at PNNL that have demonstrated the ability to measure chemical and biochemical reactions at the single-molecule level [Lu, 1997, 1998]. We have been working on extending this approach by using the surface enhanced Raman scattering (SERS) method to facilitate the application to *both fluorescent and non-fluorescent* substrate species. The strong field enhancement at a sharp AFM metal tip coupled to an external optical field of proper polarization should allow the observation of site specific SERS contrast. These novel nano-scale characterization techniques hold the promise of being able to characterize biological samples topographically and spectroscopically and investigate, at a fundamental level, the influence of system heterogeneity on the biological and chemical properties of protein-protein complexes and other biologically relevant systems. With these new techniques, we will be able to simultaneously obtain topological (nanometer resolution) and spectroscopic (a-few-wave number resolution) information, and obtain dynamic information for the same imaging area of the sample.

Another imaging method will be based upon Raman scattering, as a vibrational spectroscopy, that has been widely used in spectroscopic and chemical characterization. It has been recently demonstrated to have sensitivity sufficient to detect single molecules and provide Raman spectra for studying single molecules adsorbed on single and aggregated nanoparticles at room temperature [Michaels, 1999; Nie, 1997]. In single-molecule SERS experiments, cross sections as large 10^{-16} cm^2 per molecule (comparable with or higher than effective fluorescence cross sections) have been demonstrated in nonresonant and resonant surface-enhanced Raman scattering using visible and near infrared excitation. SERS enhancement comes from chemical enhancement (factor of 10 to 10^2) and near-field electromagnetic field enhancement (factor of 10^3 – 10^6, or even 10^{12} that was demonstrated by single-molecule SERS) [Michaels, 1999; Nie, 1997]. An AFM metal tip can be scanned in subnanometer z-range of the substrate surfaces or even in contact mode in order to fully utilize the SERS enhancement. Similar research has been reported recently [Anderson, 2002], and the results are still not sophisticated enough to show the feasibility of application. Further technical development is needed for the application of this technique to the biologically related research. We have recently developed a microscope with (far-field) SERS imaging capability and obtained preliminary SERS imaging data at single-molecule sensitivity (**Fig. 6.3.8**). The metal nano-tips are typically fabricated using electrochemical etching and high-vacuum focused-ion-beam milling of Au or Ag wires (instruments available at PNNL), or using commercial (Digital Instruments, Inc.) and home-made (We have vacuum metallic coating facilities and a focused ion-beam instrument available at PNNL) metal-coated AFM dielectric tips. The AFM metal tip-combined single-molecule/single-particle Raman spectroscopy capability holds high promises of achieving nanometer spatial resolution and single-molecule identification because the SERS enhancement is a very “local” effect.

Fig. 6.3.8. Single nanoparticle-cluster surface enhanced Raman spectroscopy and Raman imaging. 2-D Raman intensity image of the single-molecule rhodamine 6G molecules adsorbed on silver nanoparticles (514.5 nm excitation). Each feature on the image corresponds to a "hot" Raman spot. The Raman signal was detected by using an APD detector after a 30-nm-bandwidth bandpass filter at 564 nm. **(Inset, right)** Transmission electron microscope image of the silver nanoparticles used in SERS measurements. The metallic nanoparticles were synthesized by mixing the silver nitrate and sodium citrate. **(Inset, left)** 2-D Raman intensity image of the cytochrome c molecules (at 1 nM) adsorbed on silver nanoparticles at 514.5-nm excitation.



6.4 Research Design and Methods

Our general objective is to validate the existence of protein complexes within the cell. Further, we hope to elucidate the function of these complexes by providing information as to the location, dynamics and physical characteristics of the protein complex. Ultimately, we hope to provide a tool set that can provide a comprehensive description of cell metabolism on the molecular scale. To achieve these goals, we will seek to provide a comprehensive image of a cell on the molecular level. This "image" would comprise of information on the location and quantity of individual proteins, complexes and metabolites. To do this, we will pursue a multifaceted approach that involves several distinct technologies. These technologies were highlighted in Section 6.3 "Preliminary Studies" and will be refined for both intra- and extra-cellular analyses. Section 6.4.1 will focus on analyses of protein complexes in simulated environments and in living cells. Individual proteins will be prepared and combined in vitro to assess complex formation. Section 6.4.1 will also focus on the assessment of protein complexes that are located intra-cellularly. Further, measurements of the intracellular environment, such as the concentration of important metabolites, will be made. Section 6.4.2 will focus on analyses of protein complexes localized in the cellular membrane. The cell membrane represents the critical interface between the cell and its external environment. Numerous protein complexes involved in signaling and transport are located here. Efforts to identify protein complexes on live cells will be pursued.

Due to the scale of molecular/cellular systems and the importance of single complexes in this context, we will focus our analytical developments on techniques with high sensitivity, approaching the single molecule level. Such techniques are at the forefront of analytical research and will have a significant impact on molecular and cellular imaging. As discussed below, single molecule studies will aid in discerning the stoichiometry and content of complexes that cannot be obtained by bulk sample (ensemble) methods. Analysis at the single molecule level will also reduce requirements for sample preparation and enable real-time characterization of protein complexes. This will complement the "snap-shot" image provided by conventional techniques and be extremely informative as to the structure and function of protein complexes.

Research on these sub-tasks will be pursued as dictated by the requirements of the Center, the availability of funding, and the decisions of the review committee. Initial emphases will be placed on scanning probe/molecular recognition force microscopy and single molecule fluorescence techniques (such as fluorescence resonance energy transfer), described in further detail below. Several of the techniques described below can be employed in different environments and we will seek to evaluate the utility of each technical approach. Ultimately, we hope to combine appropriate analytical approaches for the simultaneous characterization of the intra- and extra-cellular environment. Initially, the research focus will be on establishment of these techniques for molecular imaging on the cellular scale. We will focus then on evolving these procedures into routine techniques and address issues related to practical implementations for high throughput characterization.

6.4.1 Studies of Protein Complexes in Isolated Complexes and Living Systems

6.4.1.1 Complexes in Isolation

Current approaches for isolating protein complexes and determining their composition rely on bulk isolation procedures. One of the proteins in the complex is "tagged" using an epitope tag or by fusing it to a secondary protein. The tagged protein and its associated partners are then isolated by affinity protocols. Alternately, antibodies are raised against one or more proteins in the complex and these antibodies are then used to selectively isolate the complex. Regardless of the initial isolation approach used, proteins within the complex are then identified by sensitive analytical techniques, such as gel electrophoresis or mass spectrometry.

The standard approach outlined above will provide some information regarding basic protein interactions in an "average" complex. It provides little or no information on the number of different complexes or the stoichometry involved. This is because the analytical techniques generally used are poorly quantitative and because of uncertainties regarding dissociation of different complex components. It is also due to the bulk nature of the isolation procedure. There are different approaches that can be used to address this problem, such as tagging multiple proteins in the complex. For example, in **Fig. 6.4.1**, standard bulk isolation approaches cannot distinguish between situations I or II. However, if proteins 2 and 3 were also tagged, then one could determine whether 2 and 3 were always isolated together (situation II) or apart (situation I). This approach has the disadvantage of being tedious (all proteins have to be eventually tagged) and does not provide information on the spatial arrangements of the proteins in the complex. It also assumes that all tagged members of the complex can be used to isolate the complex as a whole.

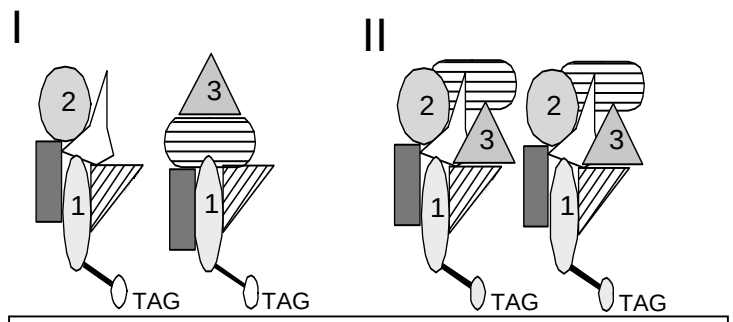


Fig. 6.4.1. An example of related (I) or identical (II) complexes that would be difficult to resolve by conventional means. Using standard isolation procedures, one cannot determine the number of different unique complexes that could form with a given tagged protein.

We propose to use an alternate approach for validating the numbers and different types of proteins in a complex, based on single molecule imaging. It requires the tagging of an initial protein in the complex, which is then used to immobilize the complex on a surface, such as one specific for 6Xhis-tagged proteins. The immobilized complexes will then be probed with labeled single-chain antibodies that are being developed as part of **t**he **High-Throughput Complex Isolation** subproject. Although these recognition agents must be developed for the different proteins in the complex, they do not have to be of sufficient affinity to isolate the complex. In addition, our approach can be used in conjunction with optical and AFM approaches to determine the topology and dynamics of the complexes.

Single-Molecule Studies of Complex Composition: Fluorescent tags will be systematically attached to pairs of proteins presumed to be in any given complex by using the methods described in 6.4.1.4. Then, each isolated complex will be probed spectroscopically to establish the coexistence (or not) of the two labeled proteins. In this way, the composition of the individual complexes can be compared to the average composition obtained through MS analysis of an aggregate sample of complexes, for example. The observed inhomogeneity will determine how many individual complexes will require analysis to obtain a statistically meaningful analysis of the distribution of complex compositions. Many of the complexes will be composed of only a few proteins. Knowing that will simplify this analysis. A three-member complex cannot have many variations in composition. However, a given protein may be involved in several different complexes, a fact that can easily be established by characterization of the range of individual complexes (one complex at a time) isolated when "pulling down" one particular protein.

The specific complexes studied will be selected in collaboration with other members of the overall project team. Whole protein complexes will be obtained from the **Center** and the **High Throughput Complex Processing**

subproject with appropriate tags for surface immobilization. Mass spectral information about the composition of the complexes will guide the use of labeling methods (Section 6.4.1.4). In some cases, purified individual proteins will be obtained from the **High Throughput Complex Processing subproject** and used to reconstitute complexes in vitro, making it easier to accomplish site-directed or at least protein specific labeling. The performance of imaging instrumentation required for these measurements is described in Section 6.3.3.1. Professor Klaus Hahn has agreed to collaborate in this work by providing dyes designed to probe the composition (and structure/function relationships, see following sections) of these complexes.

Topographic Imaging and Spectroscopic Probing of Protein Complex Interaction Dynamics: While an understanding of the complex composition will be extremely important information to obtain, much more is needed to relate existence to function. How tightly bound is the complex? How dynamic is the structure, i.e. does it remain "intact" and yet undergo large structural fluctuations? Does the addition of one particular protein to the complex have a substantial effect on the stability and flexibility of the complex? We propose the development of a combined AFM/Single Molecule Microscopy (AFM/SMM) instrument to address these questions by observing the spatial and dynamic relationships of the various proteins in a complex. A series of snapshot-AFM images of the complex structure will be obtained while observing the dynamics of point-to-point distance fluctuations using FRET.

When the distance between two chromophores is about 10 Å to 100 Å (and their respective emission and absorption spectra overlap to give a Förster energy transfer radius of about 50 Å), excited state excitation energy can transfer efficiently from one (the donor) to another (the acceptor) [Weiss, 1999; Zhuang, 2000; Lakowicz, 1992; Chen, 2002]. This is called Förster energy transfer and can be detected for a single donor/acceptor pair (spFRET) with sufficiently high sensitivity. The spFRET data provides direct information on conformational dynamics when the donor/acceptor pair are attached to a single-protein. When attached to two different proteins in single-complex, the FRET data reflect the distance between these two elements of the complex. Single complex FRET measurements will give the time dependence (on the nanosecond-to-microsecond time scale [Bustamante, 1999; Viani, 2000; Lindsay, 1989] of the distances between the points of attachment for the donor/acceptor pair while the AFM tip is used to map out the structure. The relatively slow AFM imaging rate will provide periodic snapshots of the structure as a function of time, providing two complementary views of the complex structure.

This new AFM-SMM instrument will also use the optical perturbation caused by the nearby tip to locate the position of a fluorescent marker within a complex [Lu, 2001; Novotny, 1997; Sanchez, 1999]. Depending upon the sample and tip configurations, the approach of the metallic tip within 2-10 nm of an optical chromophore can enhance the likelihood of absorption (by amplification of the local optical field and using two-photon optical excitation) and/or quench emission (by energy transfer to the tip) by an intrinsic or extrinsic optical probe molecule [Sanchez, 1999]. Tapping-mode AFM will be used for under water imaging and the expected spatial resolution for location of a chromophore is ~5nm. The metal nano-tips are typically fabricated using electrochemical etching and high-vacuum focused-ion-beam milling of Au or Ag wires (instruments available at PNNL), or using commercial (Digital Instruments, Inc.) and specially designed (Vacuum metallic coating facilities and a focused ion-beam instrument available at PNNL) metal-coated AFM dielectric tips. A significant challenge will be in the advancement of soft-materials AFM imaging at high resolution. For this purpose, cantilevers with softer spring constants should be useful. These are commercially available and will be investigated. Higher resolution imaging should also be possible with sharper probe tips. A promising approach is the growth of carbon nanotubes onto the cantilever tips [Wong, 1998]. Single walled carbon nanotubes have diameters on the order of two nanometers. Growth of such structures is possible by the techniques (i.e. chemical vapor deposition (CVD) or plasma enhanced-CVD) used to grow the vertically aligned carbon nanofibers described earlier. We have made preliminary investigations into such cantilver tips and will investigate this further. Additionally, the company, Molecular Nanosystems, promises to make these commercially available in the near term.

The single-molecule results will reveal detailed protein-protein dynamics that are unobtainable by conventional ensemble-averaged approaches due to the static and dynamic inhomogeneities and non-synchronizable conformational fluctuations of the protein-protein interactions. The methodologies of single-molecule, real-time topological/optical imaging and spectroscopy that are developed in this research will be widely applicable to

understanding the general interaction mechanisms of biological complexes, e.g., the transcription, replication, and signal transduction networks involved in many key biological functions.

Our initial objective is to develop the methodology for the application of this AFM-SMM instrument that couples a single-molecule confocal fluorescence microscope with AFM imaging using metal tips. We expect to demonstrate that the AFM-SMM instrument can locate the sites of the fluorescent interaction site within the secondary structures exhibited by individual protein complexes with an emphasis on those complexes identified as priorities by the Center research efforts. We will then use this capability to measure single-molecule protein-protein interaction time trajectories over a wide time-scale (from nanosecond to seconds) and correlate this information with structural data from the simultaneous AFM image data. Once the methodology is established, we shall focus on demonstrating the utility of the AFM-SMM instrument for use in monitoring complex formation in isolation and in cell mimics under a range of physiological conditions in real time.

Molecular Recognition Force Microscopy: Scanning probe techniques allow for molecular level, and in certain applications, atomic level descriptions of surfaces. In this task, we will employ the atomic force microscope as an alternate approach to identifying specific proteins within a complex. This technique will also enable determination of physical characteristics of the protein complex. The sensitivity of the microcantilever can be exploited to measure force interactions, such as friction and adhesion, between the probe tip and the surface. In addition, by attaching chemical species to the probe tip, interaction forces between molecular pairs can be identified. Force measurements on the order of piconewtons can be obtained allowing for measurements to be made on single molecule pairs. This has been demonstrated using antibodies [Hinterdorfer, 1996, 1998; Dammer, 1996; Allen, 1997; Willemsen, 1998], nucleic acids [Lee, 1994; Boland, 1995; Dammer, 1995], and cell recognition proteins [Fritz, 1998; Lehenkari, 1999; Chen, 2000; Baumgartner, 2000]. Recently, Prof. Hinterdorfer's group at the Johannes Kepler University in Linz, Austria has combined both topographic imaging and force interaction measurements. They have imaged lysozyme on a surface and concurrently measured the interaction between lysozyme and an antibody to lysozyme that was tethered to the cantilever probe tip. This new application of AFM is termed molecular recognition force microscopy (MRFM), and is currently being extended to imaging and identifying specific interactions on cell surfaces. Once recognition sites have been identified by topography, it is possible to move the cantilever probe to the recognition site and quantitatively measure the binding force. We are collaborating with this group to extend the MRFM technique to identifying specific reactions of enzymatic proteins with their substrates and to image cell structures (see accompanying letter from Dr. Hinterdorfer).

In this task, we will exploit the specificity and sensitivity of the MRFM technique to identify proteins within a complex. Eventually, this technique will be extended to analyses on cell surfaces, as discussed below in Section 6.4.2.1. Identification of specific complexes, their physical properties, and their distribution will be the primary focus. Dynamic information on conformational changes as a function of environmental conditions will also be sought.

Molecular recognition force microscopy is accomplished by tethering a specific functional entity to the cantilever probe tip and both imaging and identifying specific interactions with the target surface. Commercially available AFM cantilevers made of silicon or silicon nitride will be used. Advanced versions of AFM tips incorporating carbon nanotubes will also be investigated. The cantilevers will be derivatized to eventually bind the functional entity that one wishes to exploit. One approach to tip derivatization is to first bind ethanolamine to the tip. This introduces an amino group for subsequent functionalization. An alternative approach, would be to bind epoxy silane compounds to the tip, which terminate in an epoxide group, for subsequent coupling to amino compounds. In this initial step, the concentration of the reactant and reaction time can be modulated to control the number of molecules bound to the probe tip. This step is used as a means to subsequently control the extent of tip functionality [Haselgrubler., 1995]. The next step in tip derivatization involves elongation of the tether by binding a polyethylene glycol (PEG) compound to the amino or epoxide groups on the probe tip. This compound is chemically engineered to terminate in a reactive end group that will bind either substrate, proteins, enzymes, or antibodies. A variety of PEG derivatives and chain lengths can be obtained from Fluka (Milwaukee, WI or Shearwater (Huntsville, AL) to allow for introducing alternative functionality to the probe tip.

Imaging and force recognition will be accomplished using a Molecular Imaging AFM in the MacMode configuration. In order to maximize the potential for investigating the effects of environmental changes, the AFM

will be operated within an environmental chamber. In order to carry out MRFM the tip with functionality attached must be kept proximal to the sample. In MacMode, magnetically coated cantilevers are oscillated by an alternating current with both the amplitude and frequency of oscillation being controlled. This allows the tip to be oscillated at amplitudes less than the length of the 8 nm PEG tether thereby keeping the functional group in close contact with the sample. The other consideration to be addressed is that the on time (t_{on}) for the recognition interaction (i.e., antigen/antibody) must be faster than the dwell time (t_D) where the tip is over the antigen. Hinterdorfer's group found that for the lysozyme antigen/antibody reaction this is well within the normal scanning speed of 1mm/sec where they calculated t_{on} = 1ms and t_D =12ms [Raab, 1999]. Dr. Hinterdorfer's group has agreed to provide some of the unique tethers formulated in his laboratory. Initial efforts will focus on investigations of single complexes. Proteins, alone or in complexes, will be interrogated with putative binding partners. In one application, candidate proteins will be attached to the AFM tip to assess for interactions with other proteins. Longer-term efforts will investigate the utility of spatially arraying proteins for higher throughput analyses. The identification of proteins within complexes will also be investigated by binding specific recognition agents (e.g. antibodies, single chain antibodies, biotin, streptavidin) to the AFM tip and interrogating protein complexes.

6.4.1.2 Complexes Within Cells: Optical Probes for Co-Localization

Of significant importance is the validation that complexes that have been extracted from a collection of cells actually exist and function within the living cells. Has the process of extraction significantly perturbed the composition of the complexes? Are the complexes of a transient or robust nature? These and related questions require direct observations in live cells for resolution. We propose the use of high-speed measurements of optical lifetimes and rotational correlation functions to demonstrate/follow the course of protein-protein co-localization in time and space in live cells. We will develop and demonstrate the capability of measuring protein-protein interactions at ultra-high sensitivity (single-molecule sensitivity) over a wide time-scale from nanoseconds to seconds.

The determination that two proteins are co-localized and interacting cannot be determined from confocal optical imaging alone. The spatial resolution (200-500 nm) is insufficient to provide such molecular level information. We will use high-speed measurements of spFRET, lifetime and single molecule fluorescence polarization anisotropy (smFPA) to address these questions [Polakowski, 2000; Xue, 1995; Lu, 1997, 1998, 2001; Weiss, 1999; Edman, 2000; Zhuang, 2000; Macklin, 1996; Jia, 1997; Dickson, 1996; Maiti, 1997; Vanden Bout, 1997; Xie, 1999]. The spFRET signal will measure the distances between two dye labeled sites (e.g. one on each protein in a supposed complex) with a spatial resolution of 5-50 nm [Weiss, 1999; Zhuang, 2000; Lakowicz, 1992; Chen, 2002]. More importantly, as two (or more) proteins bind to form a complex, their motions will become correlated [Lu, 2001]. Thus, fluctuations in fluorescent lifetimes [Nie, 1997] (that will be modulated by local environments) and polarization anisotropy [Weiss, 1999; Cross, 1986; Lakowicz, 1992; Moerner, 1999] will become correlated reflecting not just co-localization but binding as well. This information will provide information about the stability and kinetics of complex formation/dissociation [Lu, 2001] within or on the surface of a living cell, information that is fundamental to our understanding of the biological function of particular protein complexes. For work within living cells either intrinsic fluorescence or genetically engineered labels (GFP) can be used. Also we will develop methods using nano-scale injectors for insertion of extrinsically labeled proteins into the cell.

Our state-of-the-art photon stamping (**Fig. 6.4.2**) approach will be used to measure the single-molecule fluorescence-lifetime change trajectories and the molecule anisotropy trajectories (**Fig. 6.4.3**) for the nanosecond-to-seconds time range. During this program, we shall refine our prototype photon stamping technique and demonstrate time-correlated single-photon counting and time resolved single-molecule anisotropy measurements for studies of protein-protein co-localization and conformational dynamics. We will extend our photon stamping capability by using two complementary methods: (1) A cw-laser based approach where the arrival time of each photon is "stamped" (recorded) sequentially. This approach provides the highest sensitivity and time resolution, sufficient for single-molecule studies. Every emitted photon is recorded, and subsequent off-line binning (taking an average of a certain number of channels consecutively) provides the highest time resolution, limited only by the photon flux (the excitation laser photon flux/intensity can be varied) and detector counting rate (15MHz, SPCM-AQR 16 Avalenche Photo Diode). (2) A pulsed-laser based approach where we detect each emitted photon and "stamp" its delay time, Δt, from the pump laser pulse. The delay time is the duration from the time of excitation of the single molecule to the emission by the same molecule, which is a single event measurement of the fluorescence lifetime of this molecule. The accuracy of the lifetime measurement is determined by the distribution of Δt (a Poisson distribution), and the first moment of the distribution is the lifetime. Work to further develop this technique will focus on (1) utilizing faster discriminator and computer interface hardware and software; (2) using low-dark count (<50 counts/s) photomultiplier detectors (PMT) that have better response times than APD detectors. Pulse- and cw- laser-excitation photon stamping measurements provide wide range single-molecule fluorescence dynamics: we measure the ultrafast (ps and ns) dynamics by using fs- or ps-pulse-excitation photon stamping and milisecond-to-submilisecond time-scale dynamics by using cw-excitation photon stamping.

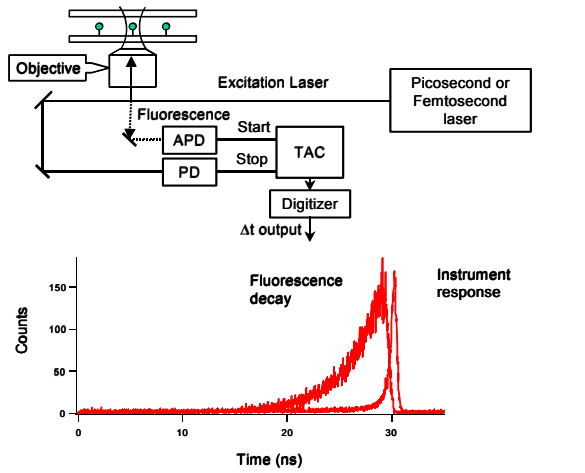


Fig. 6.4.2. Diagram of the time stamping instrument based on the traditional time-correlated single photon counting (TCSPC) approach. A picosecond or femtosecond laser is used to excite samples and to generate "start" pulses. The sample fluorescence is detected by an APD to produce "stop" pulses. The "start" and "stop" pulse times are fed into a time-to-amplitude converter. (H. LU, unpublished)

Molecule rotational motion rates are dependent upon molecular hydrodynamic shapes and masses that can be changed by molecular interactions and the formation/dissociation of a complex [Weiss, 1999; Cross, 1986; Lakowicz, 1992; Moerner, 1999]. Measuring the time-dependent anisotropy [Cross, 1986; Lakowicz, 1992] of a site-specifically labeled dye molecule can probe the conformational motions of the labeled domain of the protein [Cross, 1986; Lakowicz, 1992]. Because the time-scale for dye molecule spinning is in the sub-nanosecond range, the nanosecond-to-microsecond protein conformational motions can be differentiated and probed by measuring the longer-time-dependent anisotropy of the dye molecules. The results will be observed as changes in the fluorescence polarization anisotropy (FPA) [Weiss, 1999; Cross, 1986; Lakowicz, 1992; Moerner, 1999]. Using smFPA, we can not only measure the single-molecule domain-specific conformational changes, but also measure the substrate-protein complex formation, evolution, and dissociation in real time.

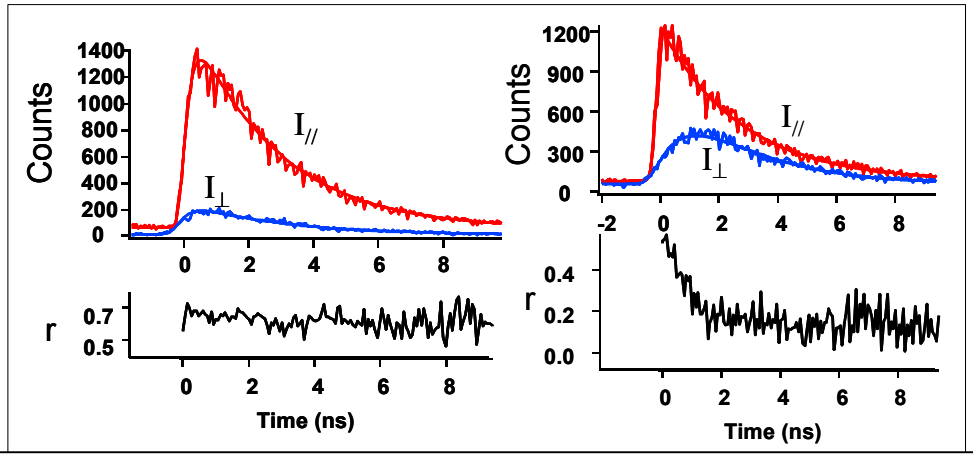


Fig. 6.4.3. Left: The time-resolved fluorescence decays $I_{//}$ and $I_{\perp}$ (top) and anisotropy $r=(I_{//}-I_{\perp})/(I_{//}+2I_{\perp})$ (bottom) of a rhodamine 123 single molecule. Rhodamine 123 single molecules are spin-coated on PMMA surface. **Right:** The time-resolved fluorescence decays $I_{//}$ and $I_{\perp}$ (top) and anisotropy $r=(I_{//}-I_{\perp})/(I_{//}+2I_{\perp})$ (bottom) of a T4 lysozyme labeled with Alexa 488. The T4 lysozyme was covalently linked to a silanized glass surface and under PBS buffer. The rhodamine123 single molecule showed no rotational motion on nanosecond timescale. The static anisotropy is due to the orientation of the single molecules' transition dipole in the laboratory frame. In contrast, T4 lysozyme with Alexa488 showed anisotropy decay due to the fast spinning of Alexa488 and slow motion of T4 lysozyme. (H. Lu, unpublished)

6.4.1.3 Intracellular Probes of Metabolic State

As described earlier, we have demonstrated the controlled synthesis and directed assembly of vertically aligned carbon nanofibers (VACNFs) into microscale and beyond structures. Using our processes, VACNF position, diameter, height, orientation and chemical composition [Merkulov, 2001a, 2001b, 2001c, 2002] can be controlled, and *individually addressable* VACNFs can be incorporated in complex microstructures using standard microfabrication techniques [Guillorn, 2001a, 2001b, 2002]. Therefore, with scalability to the wafer scale, we can deterministically fabricate nanoscale features within microscale and beyond structures compatible with cell handling.

This effort will be directed towards integrating our carbon nanofiber arrays with individual cells, both extracellularly and intracellularly. These integration methodologies will focus around traditional single-cell electrophysiological techniques such as those used for patch-clamping and interrogation with single element pulled glass capillary electrodes as well as direct cellular culture on nanofiber array substrates. We have demonstrated both direct culture of cells onto nanofiber substrates, as well as capture and micromanipulation of single cells (J774a.1, CHO, MOLT4, and Jurkat) around and onto 4-element carbon fiber arrays using traditional pulled capillary electrophysiological techniques. Briefly, cells were spun out of media and resuspended in phosphate buffered saline with 1% bovine serum albumin (to reduce short-term cell adhesion onto glass surfaces). Commercially prepared pulled capillaries were used to vacuum capture individual cells. A piezo-actuated micromanipulator was then used to position the cell with submicron resolution with respect to a 4-element fiber array grown upon an optically transparent substrate (quartz).

While carbon nanofiber probes may ultimately reveal real-time evaluation of events such as gene expression or protein complex formation in viable whole cells, this initial effort will focus on measurements of small molecule metabolites. Reactive oxygen species, for which ultramicroelectrode technology has already been demonstrated, will be initial targets. Electrodes for the measurement of substances such as nitric oxide [Malinski 1992, 1993; Brovkovych, 1999] and hydrogen peroxide [Caruana, 1999; Lacy, 1998; Garguilo, 1993] have been demonstrated and should be adaptable to carbon nanofiber probes.

For example, the detection of hydrogen peroxide at ultramicroelectrodes has been well characterized and has been a focal point within the literature for several years. Typically, single element ultramicroelectrodes (typical

diameters of 100s of nm to microns) have been used for these studies. Platinized electrodes may be employed for direct, amperometric detection of hydrogen peroxide oxidation [420 mV vs Ag/AgCl reference [Bratten, 1998], 600 mV at Pt vs Ag/AgCl reference [Lacy, 1998], or a variety of techniques may be employed to enzymatically catalyze the peroxide reduction at lower potentials to avoid potential cross-talk with other electrochemically active species within the system. A variety of these latter techniques have been reported in the literature. Caruana and Heller (1999) have employed an osmium-complexed polymeric coating on a 7 mm carbon fiber electrode coupled with soybean peroxidase (SBP) as an enzymatic catalyst to observe hydrogen peroxide reduction at -60 mV vs Ag/AgCl. Similarly, Garguilo et al. (1993) employed a redox polymer film coupled with horseradish peroxidase on a glassy carbon electrode to amperometrically quantify hydrogen peroxide within the boundary layer of the electrode. We will employ each of these techniques to evaluate which is best suited for studies with our CNF array technology.

First, we will platinize our carbon nanofiber arrays using both chemical vapor deposition (during photolithographic fabrication) and electroplating (following fabrication) to deposit a platinum coating on the exposed, electrochemically active carbon tip. We will also derivitize our CNF probes using the methods reported in literature. Briefly, we will electrophoretically deposit an electron-conducting redox polymer (PAA-PVI-Os, a 7:1 copolymer of acrylamide, acrylhydrazide, and 1-vinylimidazole complexed with Os(4,4'-dimethyl-2,2'-bipyridine)2Cl) coupled with soybean peroxidase, based upon the method described by Caruana and Heller (1999). This deposition will be characterized through cyclic voltammetry in a buffer solution and will be evaluated for performance and stability for hydrogen peroxide measurement. We will evaluate uniformity of probe response to hydrogen peroxide, determine lower detection limits, and evaluate the best cleaning method for restoring probe performance following analyte exposure.

Arrays of carbon nanofiber elements provide the ability to simultaneously probe multiple spatial locations for an analyte as well as potentially multiple analyte species. Adaptation of these features will be pursued in later years. Initial efforts will focus on standardizing measurements in vitro and eventually in vivo.

6.4.1.4 Sample Preparation and Labeling Methods

Several techniques relative to sample preparation, sample labeling and sample immobilization will be common to many of the procedures discussed above and below. Additionally, aspects of data handling and analyses will be common to all techniques. Much of this work will involve close collaboration with the other Research Thrust Areas. For example, expressed proteins and specific recognition agents will be derived from the **Center** and from the **High Throughput Complex Processing subproject.** Insertion of specific tag sequences such as those for green fluorescent protein, His-tags or streptavidin recognition will be performed also in collaboration with this subproject. Image analysis and archiving will be performed in collaboration with the **Bioinformatics and Computing subproject**. This thrust area will be also critical for structural predictions and probe design. We anticipate that these efforts will increase as the characterization techniques mature. Finally, we anticipate close collaboration with the **High Throughput Molecular Characterization subproject** on aspects related to protein cross-linking and identification of putative complexes.

Protein Immobilization: Protein immobilization onto surfaces may be necessary for analyses of isolated complexes. Protein immobilization is a commonly practiced technique, and significant prior experience has been gained with it. We have used these techniques previously for protein immobilization on silicon and other surfaces [Subramanian, 1999]. Many known immobilization strategies can be readily adapted for protein attachment to surfaces such as gold, silicon, or silicon oxide. For example, aminosilanes and aminoalkylthiol linkers can be employed to covalently tether proteins at the surface. Gold-coated surfaces can be functionalized with heterobifunctional crosslinkers [e.g., DSP (dithiobis (succinimidylproprionate))] containing thiol groups (reactive to gold) and N-hydroxy succinimide esters (reactive to primary amine groups on the protein). The carboxylic acid moieties on the C-terminus and glutamic acid and aspartic acid residues can be coupled to amine-functionalized surfaces via amide bond formation mediated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). EDC reacts with carboxylic acid groups to form a highly reactive isourea derivative that reacts with primary amines to form a peptide bond. A variety of crosslinkers are available for functionalizing gold-coated surfaces and can be prepared with thiolalkylamino crosslinkers (thiol specific to gold, amino group available for EDC coupling; e.g., dimethyl-3,3'-dithiobisproprionimidate). We will attempt to employ these, and other, conventional techniques.

However, the resulting orientation of the protein can be nonspecific, and binding properties will need to be verified.

Another approach that will be investigated employs a modified pluronic surfactant. Pluronic surfactants are tri-block copolymers (poly(ethylene oxide) – poly(propylene oxide) – poly(ethylene oxide)) that self organize onto hydrophobic surfaces [Ho, 1998]. These surfaces display extremely low nonspecific adsorption and can be used to isolated 6XHis-tagged proteins at densities appropriate for single-molecule microscopy. Integration with other specific tags (i.e. biotin) should also be facile. Directed immobilization is reversible and allows for control of protein orientation and monolayer coverage.

Protein Tagging: A number of conventional approaches to protein tagging are available and are related to the procedures discussed above, for protein immobilization. These techniques can be used for the specific attachment of fluorophores to candidate proteins. Alternate approaches involve genetic engineering and allow for specific incorporation of recognition sequences. Common examples include the use 6xHis tags and green fluorescent reporter constructs. Such reagents are commercially available. Recent advances have involved the creation of a biotin recognition sequence. Biotin is an essential coenzyme, highly conserved among species, and apparently required for all forms of life. It is active when attached to biotin carboxylases and decarboxylases that transfer CO2 and are important enzymes in gloconeogenesis, lipogenesis, aminoacid catabolism and energy transduction [Samols, 1988]. Biotinylation is an extremely rare event in cells [Cronan, 1990], and so provides a unique molecular system to specifically tag proteins with a universally competent and efficient recognition element that is also compatible with purification schemes. The binding of biotin to proteins occurs as an energy dependent (ATP utilizing) reaction to an exposed amine group usually a lysine residue. In vivo, biotinylation is specified by up to 80 aa residues. Which explains it's specificity [Chapman-Smith, 1999].

Modern molecular techniques allow the introduction of biotin accepting peptides into normal proteins, directing biotnylation to occur extrinsically [Cronan, 1990]. Biotin-fusion proteins can be created with the biotin at any site that does not disrupt protein functionality. This provides an important purification tool for biotin labeled proteins using streptavidin technology. Biotin labeled proteins can be either isolated directly, or used as a binding scaffold to detect secondary binding interactions.

Biotin accepting domains are capable of membrane translocation, making them amenable to the study of membrane proteins, [Reed, 1991; Ackerman, 1992; Jander, 1996) and can be used to tag proteins for spatial localization when observing cells in vivo or in vitro [Berliner, 1995].

Another approach to specific labeling involves the introduction of a “Strep-Tag” [Skerra, 1999]. Recent advances in strepavidin/Biotin binding technology has resulted in a customized nine-amino acid residue peptide that binds with high-affinity and specificity to strepavidin, using the biotin binding site [Skerra, 1991]. An enhanced version of the Streptag, the Strep-TagII was later selected with enhanced binding activity [Voss, 1997]. The Strep-tags belong to a growing family of affinity tags available for recombinant protein studies. Recombinant proteins containing StrepTags, can typically be purified from crude cell extracts in a single step by streptavidin affinity chromatography. The StrepTag can be placed at either end of the protein for these studies, and even internally if a suitable position can be modeled. Because of its small size, the Streptag generally does not interfere with protein folding or biological activity [Skerra, 1999]. A set of generic prokaryotic expression vectors, for production of StrepTag fusion proteins are available [Skerra, 1994].

Nanoscale Materials for Bioconjugate Imaging: Fluorescent dyes and surface-derivatized quantum dots have been used extensively in bioconjugate applications and luminescent markers or tracers [Macklin, 1996; Hermanson, 1996; Tsien, 1998a, 1998b; Lakowicz, 1992; Cha, 1999; Chan, 1998]. However, the photophysical drawbacks of molecular and quantum dot systems are well known: they are typically characterized by large spectral widths that are largely insensitive to local environments and have finite photostability. That is, a single molecule can undergo only about 10^6 absorption-emission cycles before irreversible photobleaching occurs – corresponding to an average observation time of just a few seconds under typical illumination intensity conditions. We will explore new materials with novel properties that add new dimensions to fluorescent bioconjugate applications. In particular, we will examine the use of rare-earth doped nanocrystals and luminescent polymer nanoparticles as analogs of semiconductor quantum dots. These different classes of materials each have interesting properties that can be useful for intra- and extra-cellular monitoring.

An alternative approach involves polymeric species, whose surface can be chemically tailored for specific applications. There is currently a great deal of interest in rod-shaped quantum dots that have a well-defined polarization signature and orientation. [Hu, 2000] We have recently developed a new approach to prepare polymer analogues of semiconductor quantum dots that have potentially useful fluorescent bioconjugate applications. Generated without specialized chemical synthesis techniques or templates, we are able to "print" highly monodisperse and uniformly oriented rod-shaped nanoparticles (≈ 4 – 6 nm) from microdroplets of dilute solution of a semiconducting polymer (poly phenylene vinylene (PPV) derivatives) on a glass substrate. We found that PPV nanoparticles are formed in a high-aspect ratio rod-shaped morphology, and act as *single* electric dipoles with linearly polarized emission that can be *oriented* in weak electric fields. The observation of similar behavior in polymer-blend nanoparticles (MEH-PPV and polystyrene) suggests that the MEH-PPV (normally insoluble in polar solvents) can be "capped" with another polymer to enable solubility in aqueous environments. We will investigate the synthesis and optical characterization of rare-earth doped semiconductor nanoparticles for bioconjugate applications.

6.4.2 Complexes on Cell Surfaces and Within Cell Cultures

This section focuses on the development of instruments and probes for use in understanding the role of protein complexes in enabling the function of systems of bacterial cells. Membrane bound proteins and complexes will be identified by the core elements of this overall program. The importance of imaging work will be to determine under what conditions these proteins are expressed and to gain quantitative and qualitative insight into their functions. Many bacteria exist in a synergistic relationship, communicating and exchanging metabolites via membrane and extra-cellular matrices. Imaging tools that meet the unique requirements of the system scale and complexity will be developed and applied. Some instruments described in Section 6.4.1 will have direct application. However, other tools will need to be developed for probing cell surfaces and the extra-cellular matrix. To deal with the complexity of the multi-cellular system, the focus will be on the use of multimodality instrumentation capable of simultaneously obtaining as much information as possible. Topographic maps will be accented by information about the location (and stability) of particular complexes, for example. Optical images will be obtained that are based upon multiple, non-linear signal channels, providing a level of understanding not possible with one imaging modality alone.

6.4.2.1 Multimodality AFM Imaging: Molecular Recognition Force Microscopy

The MRFM technique discussed in Section 6.4.1.5 will also be applied to analyses of cell surfaces. Specifically, AFM imaging will be used to analyze and gain molecular level information on individual wild type and mutant bacterium. Data on the number of complexes and structural changes within individual complexes will be investigated. Various substrates will be tethered to the AFM tip and used to interrogate live bacterium surfaces. The tip affinity for the substrate, the docking time, and the substrate binding forces will be measured. Further, the effect of different substrates and environmental conditions will be obtained.

The bacterial outer membrane complicates in vivo measurements. Therefore, in order to gain access to cytoplasmic membrane proteins, spheroplasts will be made by removing the outer cell membrane. This will be accomplished by employing the lysozyme EDTA method [Osborn, 1972]. Essentially, cells will be grown, pelleted, and resuspended in cold 0.75 M sucrose-10 mM Tris-HCl, pH 7.8. Lysozyme will be added to a concentration of 100 μg per ml and the mixture incubated in an ice bath for 2 minutes. Over a period of 8-10 minutes, the suspension will be diluted by the addition of cold EDTA. The EDTA solution will be added beneath the cell suspension, which will be constantly swirled in the ice bath. Conversion to spheroplasts will be monitored by phase contrast microscopy. All of the obvious variables will be adjusted in order to obtain optimal levels of spheroplasts. At this point, for further experimentation, we will either isolate intact spheroplasts or rupture the spheroplasts and isolate the cytoplasmic membrane fraction by sucrose density gradient centrifugation.

Another complication with membrane probing of live cells is identification of the proteins. Antibodies for membrane bound proteins are rare. The single chain antibodies will be useful for this application, however there is no guarantee that an exposed epitope will be recognized. Therefore, for this application we hope to exploit the use of Streptags. Streptags, introduced into proteins and expressed within the organism, are ideal substrates for MRFM. As the Streptags are small, they do not interfere with protein function, or folding, even when folded into

a membrane. Using Streptavidin-coated AFM tips [Wong, 1999], Streptags should be readily detectable on the cell surface if properly inserted. We will work with the **Bioinformatics and Computing Thrust** for identifying putative insertion regions of membrane proteins. This technique should provide actual molecular numbers on individual cells. Further, because the tags can be strategically positioned in recombinant proteins, the ability to place them on the outside, or inside of a membrane allows for assessment of protein orientation within the membrane. This powerful capability has many applications in receptor biology. We have established a collaboration with Dr. Bernhard Erni, to image the receptors for glucose on the cell surface of E. coli. These same protocols are applicable to the Rhodopsuedomonas organism, and membrane structures involved in regulating hydrogen transport, or identification of changes in Reg B/Reg A numbers, or interactions in response to environmental challenges should be possible.

6.4.2.2 Multimodality AFM Imaging: Surface Enhanced Raman Imaging

The spatial resolution afforded by AFM can be complemented with surface enhanced Raman spectroscopy (SERS) to obtain high-resolution, chemically-specific images without requiring the use of fluorescent labels. Perurbations in the interaction of the light field and the probe molecule can be caused by the near-by presence of a metal AFM tip. SERS provides large amplification of Raman signals for molecules in the close vicinity of a sharp metallic structure and shows even greater enhancement for molecules between two such structures [Xu, 1999]. With this understanding, two technical approaches can be combined to provide a new probe for extracellular probing. Gold or silver metal particles (which show high SERS gain) can be attached to antibodies, or other recognition agents, and thereby bound to particular membrane proteins for example. The molecules in the vicinity of that labeled site can then be characterized by their Raman (vibrational) spectrum at high spatial resolution by mapping the membrane surface with an AFM instrument equipped with a gold or silver tip. When the tip is in close proximity of the membrane-attached metal particles, there will be a large enhancement in the Raman signal for those molecules between the tip and the attached particle. Not only can data on location be provided (expected resolution is 2-5 nm) but the time dependence of the nature of the proteins or ligands associated with the membrane protein can be revealed by “sitting” the AFM tip at a particular site and monitoring the time dependence of the Raman signal. The first objective will be to assemble the existing components of this instrumentation into a combined instrument and demonstrated that it will provide the expected resolution and sensitivity. Antibody labeled gold nanoparticles are available. We will need to assess the importance of particle size and structure (they can be formulated as agglomerations) in controlling the signal and giving the desired enhancement in the presence of the AFM tip. This development stage should be complete in time to interface with the larger project team as they identify particular membrane complexes for investigation.

6.4.2.3 Multimodality Optical Imaging

We seek new ways to provide molecular information in living cells and for colonies of cells that are related to complex signaling behavior, particularly for extended monitoring of cellular and extra-cellular changes that are a consequence of cell signaling. Establishing connections between cellular molecular biology and extant system properties requires information over a wide range of size and time scales using live systems. Combining traditional two-photon imaging with simultaneous detection of Coherent Antistokes Raman (CARS), Second Harmonic Generation (SHG) and Sum-Frequency Generation (SFG) image detection [Mukamel,1995; Shen,1984; Squier, 2001; Chu, 2001] will provide a wealth of information not otherwise available about the complex processes occurring within (and between) cells.

Traditional methods often rely upon sampling, fixing, sectioning and staining which, of course, presents a static view of a particular biology system state. Dynamic measurements using in situ fluorescence labeling is useful but specific probes (e.g. using antibody labeling) are not easily applied to the interior of live bacterial cells, and cell permeable stains are relatively nonspecific as well as subject to bleaching and phototoxicity effects. The CARS method utilizes vibrational, not electronic, properties of molecules and thereby overcomes photo-induced effects while providing molecular information. SHG imaging [Shen,1984; Squier, 2001; Chu, 2001] is based on physical properties of interfaces and is particularly useful for extended structures such as extracellular matrices. SFG is also sensitive to interface structures and can contain molecule specific information [Shen,1984] , if one of the frequencies is resonant with a vibrational frequency of a molecule at the interface, for example. A substantial

advance would result from the combination of SHG, SFG and CARS imaging simultaneously with fluorescence by two-photon confocal (TPC) detection, providing a multidimensional image capability that reports a wide range of cell system characteristics. Understanding how to obtain and extract information from the rich data provide by this approach is the subject of the first phase of the proposed. Development of this system into a routine tool will be challenging, yet the potential is substantial. We have succeeded in combining CARS and TPC and are modifying the microscope to detect the other signal channels as well. Fortunately, the same laser system can produce all four channels with a judicious choice of laser parameters. To our knowledge, no one has attempted to combine all of these capabilities into one instrument platform. Parallel efforts are underway to improve the fundamental laser and optical technologies, which will be widely applicable for this work as well as all multiphoton microscopy methods.

These techniques offer improved chemical specificity (such as Raman signatures of specific biomolecules) and enhanced spatial resolution. As shown by Webb and co-workers, a significant enhancement in spatial resolution can be realized in any nonlinear excitation process that scales as some power of the electric field. In the case of 2-photon laser-induced fluorescence, where near-infrared photons are used as an excitation source, a reduction in background signals (due primarily to Raman scattering [Zumbusch, 1999; Volkmer, 2001] from the solvent) is also achieved. One approach that has interesting potential for cellular monitoring applications are sum-frequency generation (SFG) measurements (either degenerate or nondegenerate) for probing specific biomolecules within the cell membrane. Since the SFG signal requires a non-centrosymmetric local environment, it is an ideal probe for species located near the cell boundary. This nonlinear optical tool has been used extensively for characterizing thin films and species produced during epitaxial film growth because of its sensitivity to molecules located at the surface. SFG probes of thin-film chemical properties are facilitated in large part by the fact that the phase-matching angle (for conservation of momentum in the wave-mixing process) is well-defined for a two-dimensional system and the scattered beam is spatially separated from the input beams. This is not the case for curved surfaces, in particular where the radius of curvature is comparable to the probe wavelength, where the SFG beam is scattered in multiple angles thus 'diluting' the signal. Thus SFG has seen limited applicability in probing surfaces of quasi-spherical particles such as individual cells.

SFG, SHG and other nonlinear optical probes are useful [Shen,1984; Squier, 2001; Chu, 2001] and largely unexplored tools for probing nanoscopic species within cells. The probe wavelengths can be tuned to match specific target biomolecules such as proteins, etc. We will develop techniques for probing structure and dynamics of these species near the surface of cells; in combination with AFM/MRFM scanning probe techniques, this should provide an unparalleled view of cellular activity near the surface.

6.5 Sub-Contract and Consortium Arrangements

Dr. Peter R. Hoyt, a molecular geneticist with The University of Tennessee, has 20 years experience working with gene and protein expression in cells. He is experienced in scanning probe microscopy, fluorescence microscopy, electron microscopy, and optical microscopes to image cellular structures and single molecules. His contributions will include the production of samples for imaging, and development of imaging-compatible in vitro systems.

6.6 Literature Cited

Abe, T., Lau, Y.Y., and Ewing, A.G., "Intracellular Analysis with an Immobilized-Enzyme Glucose Electrode Having a 2µm Diameter and Subsecond Response Times", J. Am. Chem. Soc., 113, 7421 (1991).

Ackerman, S.H., Martin, J., Tzagoloff, A., " Characterization of ATP11 and detection of the encoded protein in mitochondria of Saccharomyces cerevisiae.", J. Biol. Chem., 267, 7386 (1992).

Allen, S., Chen, X., Davies, J., Davies, M.C., Dawkes, A.C., Edwards, J.C., Roberts, C.J., Sefton, J., Tendler, S.J.B., and Williams, P.M., "Detection of Antigen-Antibody Binding Events with the Atomic Force Microscope Biochemistry", 36(24), 7457 (1997).

Allison, D.P. Kerper, P.S., Doktycz, M.J., Thundat, T., Modrich, P., Larimer, F.W., Johnson, D.K., Hoyt, P.R., Mucenski, M.L., and Warmack, R.J., "Mapping Individual Cosmid DNAs by Direct AFM Imaging", Genomics, 41, 379 (1997).

Allison, D.P., Kerper, P.S., Doktycz, M.J., Spain, J.A., Modrich, P., Larimer, F.W., Thundat, T., and Warmack, R.J., "Direct Atomic-Force Microscope Imaging of EcoRI Endonuclease," Proc. Natl. Acad. Sci., 93(17), 8826 (1996).

Anderson, M.S. and Pike, W.T., "A Raman-Atomic Force Microscope for Apertureless-Near-Field Spectroscopy and Optical Trapping", Rev. Sci. Inst., 73, 1198 (2002).

Barnes, M.D., Mehta, A., Thundat, T., Chaabra, B., Kulkarkni, S., and Bhargava, R.N., "On-Off Blinking and Multiple Bright States of Single Europium Ions in Eu3+:Y203 Doped Nanocrystals", J. Phys. Chem., B104, 6099 (2000).

Baumgartner, W., Hinterdorfer, P., Ness, W., Raab, A., Vestweber, D., Schindler, H., and Drenckhahn, D., "Cadherin Interaction Probed by Atomic Force Microscopy", Proceedings of the National Academy of Sciences of the United States of America, 97, 4005 (2000).

Berliner, E., Young, E.C., Anderson, K., Mahtani, H.K., Gelles, J., " Failure of a Single-Headed Kinesin to Track Parallel to Microtubule Protofilaments.", Nature, 373, 718 (1995).

Binnig, G., Quate, C.F., and Gerber, C., "Atomic Force Microscope", Physical Review Letters, 56, 930 (1986).

Boland, T. and Ratner, B.D., "Direct Measurement of Hydrogen Bonding in DNA Nucleotide Bases by Atomic Force Microscopy", Proc. Natl. Acad. Sci. USA, 92, 5297 (1995).

Bolshakova, A.V., Kiselyova, O.I., Filonov. A.S., Frolova, O.Y., Lyubchenko, Y.L., and Yaminsky, I.V., "Comparative Studies of Bacteria With an Atomic Force Microscope Operating in Different Modes", Ultramicroscopy, 86, 121 (2001).

Bratten, C.D., Cobbold, P.H., and Cooper, J.H., "Single-Cell Measurements of Purine Release Using a Micromachined Electroanalytical Sensor", Anal. Chem., 70(6), 1164 (1998).

Brovkovych, V., Stolarczyk, E., Oman, J., Tomboulian, P., and Malinski, T., "Direct Electrochemical Measurement of Nitric Oxide in Vascular Epithelium", J. Pharm. Biomed. Anal., 19, 135 (1999).

Bruchez Jr., M., Moronne, M., Gin, P., Weiss, S., and Alivisatos, A.P., "Semiconductor Nanocrystals as Fluorescent Biological Labels", Science, 281, 2013 (1998).

Bustamante, C., Guthold, M., Zhu, X., and Yang, G., "Facilitated Target Location on DNA by Individual Escherichia coli RNA Polymerase Molecules Observed with the Scanning Force Microscope Operating in Liquid", J. Biol. Chem., 274**,** 16665 (1999).

Caruana, D.J. and Heller, A., "Enzyme-Amplified Amperometric Detectino of Hybridization and of a Single Base Pair Mutation in an 18-base Oligonucleotide on a 7-mu m-diameter Microelectrode", J. Am. Chem. Soc., 121(4), 769 (1999).

Cha, A., Snyder, G.E., Selvin, P.R., and Bezanilla, F., "Atomic Scale Movement of the Voltage-Sensing Region in a Potassium Channel Measured via Spectroscopy", Nature, 402, 809 (1999).

Chan, W.C. and Nie, S., "Quantum Dot Bioconjugates for Ultrasensitive Nonisotopic Detection", Science, 281, 2016 (1998).

Chapman-Smith, A., Cronan, J.E. Jr., "In Vivo Enzymatic Protein Biotinylation", Biomolec. Engineering, 16, 119 (1999).

Chen, A. and Moy, V.T., "Cross-Linking of Cell Surface Receptors Enhances Cooperativity of Molecular Adhesion", Biophys. J., 78, 2814 (2000).

Chen, Y., Hu, D., Vorpegal, E., and Lu, H.P., "Probing of Single-protein Conformational Dynamics under Enzymatic Reaction in Real-time", submitted to Proc. Natl. Acad. Sci., (2002).

Cheng, J-X., Volkmer, A., Book, L.D., and Xie, X.S., "An Epi-Detected Coherent Anti-Stokes Raman Scattering (E-CARS) Microscope with High Spectral Resolution and High Sensitivity", J. Phys. Chem. B, 105, 1277 (2001).

Cheng, J-X., Book, L.D., and Xie, X.S., "Polarization Coherent Anti-Stokes Raman Scattering Microscopy", Opt. Lett., 26, 1341 (2001).

Chu, S-W., Chen, I-H., Liu, T-M., Chen, P.C., and Sun, C-K., "Multimodal Nonlinear Spectral Microscopy Based on a Femtosecond Cr:forsterite Laser," Opt. Lett., 26, 1909 (2001).

Cronan, J.E., "Biotination of proteins in vivo. A post-translational modification to label, purify, and study proteins", J. Biol. Chem., 265, 10327 (1990).

Cross, A.J. and Fleming, G.R., "Influence of Inhibitor Binding on the Internal Motions of Lysozyme", Biophys. J., 50**,** 507 (1986).

Dammer, U., Popescu, O., Wagner, P., Anselmetti, D., Guntherodt, H.-J., and Misevic, G.N., "Binding Strength Between Cell Adhesion Proteoglycans Measured by Atomic Force Microscopy", Science, 267, 1173 (1995).

Dammer, U., Hegner, M., Anselmetti, D., Wagner, P., Dreier, M., Huber, W., and Guntherodt, H.-J., "Specific Antigen/Antibody Interactions Measured by Force Microscopy", Biophys. J., 70, 2437 (1996).

Dickson, R.M., Norris, D.J., Tzeng, Y.L., and Moerner, W.E., "Three-Dimensional Imaging of Single Molecules Solvated in Pores of Poly(acrylamide) Gels", Science, 274**,** 966 (1996).

Edman, L. and Rigler, R., "Memory Landscapes of Single-Enzyme Molecules", Proc. Natl. Acad. Sci. USA, 97**,** 8266 (2000).

Florin, E.L., Moy, V.T., and Gaub, H.E., "Adhesion Forces Between Individual Ligand-Receptor Pairs", Science, 264, 415 (1994).

Fritz, J., Katopidis, A.G., Kolbinger, F., and Anselmetti, D., "Force-Mediated Kinetics of Single P-selectin/ligand Complexes Observed by Atomic Force Microscopy", Proc. Natl. Acad. Sci. USA, 95, 12283 (1998).

Garguilo, M.G., Huynh, N., Proctor, A., Michael, A.C., "Amperometric Sensors for Peroxide, Choline, and Acetylcholin Based on Electron Transfer Between Horseradish Peroxidase and a Redox Polymer", Anal. Chem., 65(5), 523 (1993).

Guillorn, M.A., Ellis, E.D., Simpson, M.L., Merkulov, V.I., Melechko, A.V., Baylor, L.R., Bordonaro, G.J., and Lowndes, D.H., "Microfabricated Field Emission Devices Using Carbon Nanofibers as Cathode Elements", J. Vac. Sci. Tech. B., 19(6), 2598 (2001).

Guillorn, M.A., Ellis, E.D., Britton, C.L., Simpson, M.L., Melechko, A.V., Merkulov, V.I., Bordonaro, G.J., Woodie, D., Baylor, L.R., and Lowndes, D.H., "Operation of a Gated Field Emitter Using an Individual Carbon Nanofiber Cathode", Appl. Phys. Lett., 79(21), 3506 (2001).

Guillorn, M.A., McKnight, T.E., Melechko, A., Merkulov, V.I., Austin, D.W., Lowndes, D.H., and Simpson, M.L., "Individually Addressable Vertically Aligned Carbon Nanofiber-Based Electrochemical Probes", J. Appl. Phys., 91(6), 3824 (2002).

Harms, G., Orr, G., Montal, M., Thrall, B., Colson, S., and Lu, H.P., "Probing Conformational Dynamics of a Single Ion Channel in Real Time", submitted to Nature (2002).

Haselgrubler, T., Amerstorfer, A., Schindler, H., and Gruber, H., "Synthesis and Applications of a New Poly(ethylene glycol) Derivative for the Cross-Linking of Amines with Thiols", Bioconjugate Chemistry, 6, 242 (1995).

Henderson, E., Haydon, P.G., and Sakaguchi, D.S., "Actin Filament Dynamics in Living Glial Cells Imaged by Atomic Force Microscopy", Science, 257, 1944 (1992).

Hermanson, G.T., Bioconjugate Techniques, Academic Press, New York, 1996.

Hinterdorfer, P., Baumgartner, W., Gruber, H.J., Schilcher, K., and Schindler, H., "Detection and Localization of Individual Antibody-Antigen Recognition Events by Atomic Force Microscopy", Proc. Natl. Acad. Sci. U.S.A, 93, 3477 (1996).

Hinterdorfer, P., Schilcher, K., Baumgartner, W., Gruber, H.J., and Schindler, H., "A Mechanistic Study of the Dissociation of Single Antibody-Antigen Pairs by Atomic Force Microscopy", Nanobiology, 4, 177 (1998).

Ho, C.-H., Limberis, L., Caldwell, K.D., and Stewart, R.J., "A Metal-Chelating Pluronic for Immobilization of Histidine-Tagged Proteins at Interfaces: Immobilization of Firefly Luciferase on Polystyrene Beads", Langmuir, 14, 3889 (1998).

Hoh, J.H., Cleveland, J.P., Prater, C.B., Revel, J-P., and Hansma, P.K., "Quantized Adhesion Detected with the Atomic Force Microscope", J. Am. Chem. Soc., 114, 4917 (1992).

Hoh, J.H. and Schoenenberger, C.A., "Surface Morphology and Mechanical Properties of MDCK Monolayers by Atomic Force Microscopy", J. Cell Science, 107, 1105 (1994).

Holtom, G.R., Thrall, B.D., Chin, B-Y., Wiley, H.S., and Colson, S.D., "Achieving Molecular Selectivity in Imaging Using Multiphoton Raman Spectroscopy Techniques", Traffic, 2, 781 (2001).

Holtom, G., Thrall, B., Weber, T., Zhu, L., Hopkins, D., Parkinson, C., Colson, S., Price, J., Chin, B-Y., Choi, A., and Risby, T., "Application of Coherent Anti-Stokes Raman Scattering (CARS) to Imaging Mammalian Cells: A Means for Gaining Molecular Selectivity in Multiphoton Imaging", Proc. of SPIE 4262, edited by Ammasi Periasamy and Peter T. C. So, 319 (2001).

Hoyt, P.R., Doktycz, M.J., Warmack, R.J., and Allison, D.P., "Spin Column Isolation of Protein-DNA Interactions from Complex Protein Mixtures for AFM Imaging", Ultramicroscopy, 86, 139 (2001).

Hoyt, P.R., Doktycz, M.J., Modrich, P., Warmack, R.J., and Allison, D.P., "Identifying Sequence Similarities Between DNA Molecules", Ultramicroscopy, 82, 237 (2000).

Hu, J., Li, L.-S., Yang, W., Mannam, L., Wang, L.-W., and Alivisatos, A.P., "Linear Polarized Emission from Colloidal Semiconductor Quantum Rods", Science, 292, 2060 (2000).

Huang, L. and Kennedy, R.T., "Exploring Single-Cell Dynamics Using Chemically-Modified Electrodes", Trends Anal. Chem., 14(4), 158 (1995a).

Huang, L., Shen, H., Atkinson, M.A., and Kennedy, R.T., "Detection of Exocytosis at Individual Pancreatic Beta-Cells by Amperometry at a Chemically-Modified Electrode", P. Natl. Acad. Sci. USA, 92(21), 9608 (1995b).

Jander, G., Cronan, J.E., Beckwith, J., " Biotinylation in vivo as a sensitive indicator of protein secretion and membrane protein insertion ", J. Bacteriol., 178, 3049 (1996).

Jia, Y., Sytnik, A., Li, L., Vladimirov, S., Cooperman, B.S., and Hochstrasser, R.M., "Nonexponential Kinetics of a Single tRNAPhe Molecule under Physiological Conditions", Proc. Natl. Acad. Sci. USA, 94**,** 7932 (1997).

Kennedy, R.T., Huang, L., Atkinson, M.A., and Dush, P., "Amperometric Monitoring of Chemical Secretions from Individual Pancreatic Beta-Cells", Anal. Chem., 65, 1882 (1993).

Lacy, F., Gough, D.A., and Schmid-Schonbein, G.W., "Role of Xanthine Oxidase in Hydrogen Peroxide Production", Free Radic. Biol. Med., 25(6), 720 (1998).

Lakowicz, J.R., Topics in Fluorescence Spectroscopy: Biochemical Applications, Vol. 3, Plenum Press, New York, 1992.

Lee, G.U., Chrisey, L.A., and Colton, R.J., "Direct Measurement of the Forces Between Complementary Strands of DNA", Science, 266, 771 (1994a).

Lee, G.U., Kidwell, D.A., and Colton, R.J., "Sensing Discrete Streptavidin Biotin Interactions With Atomic-Force Microscopy", Langmuir, 10, 354 (1994b).

LeGrimellec, C., Lesniewska, E., Cachia, C., Schreiber, J.P., de Fornel, F., and Goudonnet, J.P., "Imaging of the Membrane-Surface of MDCK Cells by Atomic Force Microscopy", Biophysical Journal, 67, 36 (1994).

LeGrimellec, C., Lesniewska, E., Giocondi, M.C., Finot, E., and Goudonnet, J.P., "Simultaneous Imaging of the Surface and the Submembraneous Cytoskeleton in Living Cells by Tapping Mode Atomic Force Microscopy", CR ACAD SCI III-VIE, 320, 637 (1997).

Lehenkari, P.P. and Horton, M.A., "Single Integrin Molecule Adhesion Forces in Intact Cells Measured by Atomic Force Microscopy", Biochem. Biophys. Res. Comm., 259, 645 (1999).

Lindsay, S.M., Thundat, T., Nagahara, L., Knipping, U., and Rill, R.L., "Images of the DNA Double Helix in Water", Science, 244**,** 1063 (1989).

Lu, H.P. and Xie, X.S., "Single-Molecule Spectral Fluctuations at Room Temperature", Nature, 385**,** 143 (1997).

Lu, H.P., Xun, L., and Xie, X.S., "Single-Molecule Enzymatic Dynamics", Science, 282**,** 1877 (1998).

Lu, H.P., Iakoucheva, L.M., and Ackerman, E.J., Single-Molecule Conformational Dynamics of Fluctuating Noncovalent DNA - Protein Interactions in DNA Damage Recognition", J. Am. Chem. Soc., 123**,** 9184 (2001).

Ludwig, M., Moy, V.T., Rief, M., Florin, E.L., and Gaub, H.E., "Characterization of the Adhesion Force Between Avidin- Functionalized Afm Tips and Biotinylated Agarose Beads", Microscopy Microanalysis Microstructures, 5, 321 (1994).

Macklin, J.J., Trautman, J.T., Harris, T.D., and Brus, L.E., "Imaging and Time-Resolved Spectroscopy of Single Molecules at an Interface", Science, 272**,** 255 (1996).

Maiti, S., Haupts, U., and Webb, W.W., "Fluorescence Correlation Spectroscopy: Diagnostics for Sparse Molecules", Proc. Natl. Acad. Sci. USA, 94**,** 11753 (1997).

Malinski, T. and Taha, Z., "Nitric Oxide Release from a Single Cell Measured in situ by a Porphyrinic-Based Microsensor", Nature, 358, 676 (1992).

Malinski, T. and Taha, Z., "Measurements of Nitric Oxide in Biological Materials Using a Porphyrinic Microsensor", Anal. Chim. Acta, 279, 135 (1993).

Matthews, B.W., "Studies On Protein Stability With T4 Lysozyme", Advances in Protein Chemistry, 46**,** 249 (1995).

Merkulov, V.I., Melechko, A.V., Guillorn, M.A., Lowndes, D.H., and Simpson, M.L., "Effects of Spatial Separation on the Growth of Vertically Aligned Carbon Nanofibers Produced by Plasma-Enhanced Chemical Vapor Deposition", Appl. Phys. Lett., 80(3), 476 (2002).

Merkulov, V.I., Melechko, A.V., Guillorn, M.A., Lowndes, D.H., and Simpson, M.L., "Sharpening of Carbon Nanocone Tips During Plasma-Enhanced Chemical Vapor Growth", Chem. Phys. Letts., 350(5-6), 381 (2001).

Merkulov, V.I., Melechko, A.V., Guillorn, M.A., Lowndes, D.H., and Simpson, M.L., "Alignment Mechanism of Carbon Nanofibers Produced by Plasma-Enhanced Chemical-Vapor Deposition", Appl. Phys. Lett., 79(18), 2970 (2001).

Merkulov, V.I., Guillorn, M.A., Lowndes, D.H., Simpson, M.L., and Voelkl, "Shaping Carbon Nanostructures by Controlling the Synthesis Process", Appl. Phys. Lett., 79(8), 1178 (2001).

Michaels, A.M., Nirmal, M., and Brus, L.E., "Surface Enhanced Raman Spectroscopy of Individual Rhodamine 6G Molecules on Large Ag Nanocrystals", J. Am. Chem. Soc., 121, 9932 (1999).

Moerner, W.E. and Orrit, M., "Illuminating Molecules in Condensed Matter", Science, 283, 1670 (1999).

Mukamel, S., Principles of Nonlinear Optical Spectroscopy, Oxford University Press, 1995.

Muller, D.J., Baumeister, W., and Engel, A., "Controlled Unzipping of a Bacterial Surface Layer with Atomic Force Microscopy", Proceedings of the National Academy of Sciences of the United States of America, 96, 13170 (1999).

Netchiporouk, L.I., Shram, N.F., Jaffrezic-Renault, N., Martelet, C., and Cespuglio, R., "In Vivo Brain Glucose Measurements: Differential Normal Pulse Voltammetry with Enzyme-Modified Carbon Fiber Microelectrodes", Anal. Chem., 68, 4358 (1996).

Nie, S. and Emory, S.R., "Probing Single Molecules and Single Nanoparticles by Surface-Enhanced Raman Scattering", Science, 275, 1102 (1997).

Novotny, L., Bian, R.X., and Xie, X.S., "Theory of Nanometric Optical Tweezers", Phys. Rev. Lett., 79**,** 645 (1997).

Oberhauser, A.F., Marszalek, P.E., Erickson, H.P., and Fernandez, J.M., "The Molecular Elasticity of the Extracellular Matrix Protein Tenascin", Nature, 393, 181 (1998).

Oesterhelt, F., Oesterhelt, D., Pfeiffer, M., Engel, A., Gaub, H.E., and Muller, D.J., "Unfolding Pathways of Individual Bacteriorhodopsins", Science, 288, 143 (2000).

Osborn, M.J., Gander, J.E., Parisi, E., and Carson, J., "Mechanism of Assembly of the Outer Membrane of Salmonella typhimurium", J. Biol. Chem., 247, 3962 (1972).

Polakowski, D.B., Skelley, C.A., and Dovichi, N.J., "Single Molecules of Highly Purified Bacterial Alkaline Phosphatase Have Identical Activity", J. Am. Chem. Soc., 122**,** 4853 (2000).

Raab, A., Han, W.H., Badt, D., Smith-Gill, S.J., Lindsay, S.M., Schindler, H., and Hinterdorfer, P., "Antibody Recognition Imaging by Force Microscopy", Nature Biotechnology, 17, 902 (1999).

Reed, K.E., Cronan, J.E., " Escherichia coli Exports Previously Folded and Biotinated Protein Domains.", J. Biol. Chem., 266, 11425 (1991).

Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J.M., and Gaub, H.E., "Reversible Unfolding of Individual Titin Immunoglobulin Domains by AFM", Science, 276, 1109 (1997).

Rotsch, C. and Radmacher, M., "Drug-Induced Changes of Cytoskeletal Structure and Mechanics in Fibroblasts: An Atomic Force Microscopy Study", Biophysical Journal, 78, 520 (2000).

Samols, D., Thorton, C.G., Murtif, V.L., Kumar, G.K., Haase, F.C., Wood, H.G., " Evolutionary Conservation Among Biotin Enzymes ", J. Biol. Chem., 263, 6461 (1988).

Sanchez, E.J., Novotny, L., and Xie, X.S., "Near-field Fluoescence Microscopy Based on Two-photon Excitation with Metal Tips", Phys. Rev. Lett., 82**,** 4014 (1999).

Shen, Y. R.,The Principles of Nonlinear Optics, John Wiley & Sons, 1984.

Skerra, A., Dreher, M., Winter, G., " Filter Screening of Antibody Fab Fragments Secreted From Individual Bacterial Colonies: Specific Detection of Antigen Binding with a Two-Membrane System.", Anal. Biochem., 196, 151 (1991).

Skerra, A. and Schmidt, T.G.M., "Applications of a Peptide Ligand for Streptavidin: The Strep-Tag", Biomolec. Engineering, 16, 79 (1999).

Skerra, A., " Use of the Tetracycline Promoter for the Tightly Regulated Production of a Murine Antibody Fragment in Escherichia coli.", Gene, 151, 131 (1994).

Smith, J. and Modrich, P., "Mutation Detection with MutH, MutL, and MutS Mismatch Repair Proteins", Proc. Natl. Acad. Sci., 93, 4374 (1996).

Squier, J., Muller, M., "High Resolution Nonlinear Microscopy: A Review of Sources and Methods for Achieving Optimal Imaging," Rev. of Scientific Instruments, 72, 2855 (2001).

Subramanian, A., Kennel, S.J., Oden, P.I., Jacobson, K.B., Woodward, J., and Doktycz, M.J., "Comparison of Techniques for Enzyme Immobilization on Silicon Supports", Enzyme and Microbial Technology, 24, 26 (1999).

Sukolov, I.Y., Firtel, M., and Henderson, G.S., "In situ High-Resolution Atomic Force Microscope Imaging of Biological Surfaces", J. Vac. Sci. Technol., 14, 674 (1996).

Thomson, N.H., Fritz, M., Radmacher, M., Cleveland, J.P., Schmidt, C.F., and Hansma, P.K., "Protein Tracking and Detection of Protein Motion Using Atomic Force Microscopy", Biophys. J., 70(5), 2421 (1996).

Tsien, R.Y. and Miyawaki, A., "Seeing the Machinery of Live Cells", Science, 280, 1954 (1998).

Tsien, R.Y., "The Green Fluorescent Protein," Ann. Rev. Biochem., 67, 509 (1998).

Vanden Bout, D.A., Yip, W.T., Hu, D., Fu, D.K., Swager, T.M., and Barbara, P.F., "Discrete Intensity Jump and Intramolecular Electronic Energy Transfer in the Spectroscopy of Single Conjugated Polymer Molecules", Science, 277**,** 1074 (1997).

Viani, M.B., Pietrasanta, L.I., Thompson, J.B., Chand, A., Gebeshuber, I.C., Kindt, J.H., Richter, M., Hansma, H.G., and Hansma, P.K., "Probing Protein-Protein Interactions in Real Time", Nature Structural Biology, 7**,** 644 (2000).

Volkmer, A., Book, L.D., and Xie, X.S., "Time-resolved Coherent Anti-Stokes Raman Scattering Microscopy: Imaging Based on Raman Free Induction Decay", Appl. Phys. Lett., 80, 1505 (2002).

Volkmer, A., Cheng, J-X., and Xie, X.S., "Vibrational Imaging with High Sensitivity via Epidetected Coherent Anti-Stokes Raman Scattering Microscopy", Phys. Rev. Lett., 87*,* 03901 (2001).

Voss, S. and Skerra, A., " Mutagenesis of a Flexible Loop in Streptavidin Leads to Higher Affinity for the Strep-tag II Peptide and Improved Performance in Recombinant Protein Purification.", Protein Engineering, 10, 975 (1997).

Wagner, R., Debbie, P., and Radman, M., "Mutation Detection Using Immobilized Mismatch Binding Protein (MutS)", Nucl. Acids Res., 23(19), 3944 (1995).

Wang, M.D., Yin, H., Landick, R., Gelles, J., and Block, S.M., "Stretching DNA with Optical Tweezers", Biophys. J., 72, 1335 (1997).

Weiss, S., "Fluorescence Spectroscopy of Single Biomolecules", Science, 283**,** 1676 (1999).

Willemsen, O.H., Snel, M.M.E., van der Werf, K.O., de Grooth, B.G., Greve, J., Hinterdorfer, P., Gruber, H.J., Schindler, H., van Kooyk, Y., and Figdor, C.G., "Simultaneous Height and Adhesion Imaging of Antibody-Antigen Interactions by Atomic Force Microscopy", Biophys. J., 75, 2220 (1998).

Wong, S.S., Joselevich, E., Woolley, A.T., Cheung, C.L., and Lieber, C.M., "Covalently Functionalized Nanotubes as Nanometre-sized Probes in Chemistry and Biology", Nature, 394, 52 (1998).

Wong, J., Chilkoti, A., Moy, V.T., "Direct Force Measurements of the Streptavidin-Biotin Interaction", Biomolec. Eng., 16, 45 (1999).

Wyman, C., Rombel, I., North, A.K., Bustamante, C., and Kustu, S., "Unusual Oligomerization Required for Activity of NtrC, a Bacterial Enhancer-Binding Protein", Science, 275, 2658 (1997).

Xie, X.S. and Lu, H.P., "Single-Molecule Enzymology", J. Biol. Chem., 274**,** 15967 (1999).

Xu, H., Bjerneld, E. J., Kall, M., Borjesson, L., "Spectroscopy of Single Hemoglobin Molecules by Surface Enhanced Raman Scattering", Phys. Rev. Lett., 21, 4357 (1999)

Xue, Q. and Yeung, E.S., "Differences in the Chemical Reactivity of Individual Molecules of an Enzyme", Nature, 373**,** 681 (1995).

Zhuang, X.W., Bartley, L.E., Babcock, H.P., Russell, R., Ha, T.J., Herschlag, D., and Chu, S., "A Single-Molecule Study of RNA Catalysis and Folding", Science, 288**,** 2048 (2000).

Zumbusch, A., Holtom, G.R., and Xie, X.S., "Coherent Anti-Stokes Raman Scattering Microscopy", Phys. Rev. Lett., 82*,* 4142 (1999).

Zumbusch, A., Holtom, G.R., and Xie, X.S., "Vibrational Microscopy Using Coherent Anti-stokes Raman Scattering", Phys. Rev. Lett., 82, 4014 (1999).

6.8 Milestones

As described in the Overview to the Center, the molecular and cellular imaging tasks will start out at a lower funding level, with expected increases in the FY04 and FY05 budgets. Emphases on particular sub-tasks of the Molecular and Cellular Imaging proposal will depend on the maturity of individual techniques and their relevance to the needs of the Center. We expect that the availability of funds and the decisions of the advisory panel will shape the specifics of the research tasks.

Initial emphases will be placed on the application of molecular recognition force microscopy and single molecule FRET as described in **Sections 6.4.1.1** and **6.4.2.1**. The development of these tools for assessment of isolated complexes and living systems will be pursued. AFM and MRFM images of lives cells and isolated complexes are expected in FY03. Expected deliverables for spFRET include stoichiometry measurements of several isolated complexes. Investigations into the extension of this technique for in vivo use will also be pursued.

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