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Enclosure 1

Probing and manipulating protein conformation changes by time-resolved single-molecule spectroscopy and site-specific ultramicroscopy

Final Report (W911NF-06-1-0337)

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Introduction

This project was to develop and demonstrate a novel single-molecule spectroscopy and imaging technique to support the DARPA program on Control of Protein Conformations. The goal of our project is to advance, integrate, and apply cutting-edge atomic force microscopy (AFM) and single-molecule imaging techniques to control and analyze the protein conformational changes and according activity changes through AFM force manipulation and fluorescence resonant energy transfer (FRET) measurements as a function of local site geometry and molecular structure. In this project, we have focused our demonstration and study on controlling protein conformations to manipulate the enzyme reactivity, affinity, and selectivity. Our approach has been to use AFM tip to control protein conformational states and to apply single-molecule FRET imaging to measure the protein enzyme activity in real time. Our progress towards the demonstration of the new technology has provided an unprecedented advancement on a high spatially (nanometers) and temporally (μs to seconds) resolved single-molecule spectroscopy of optically and mechanically controlling single-molecule protein conformational changes and activities.

Research Achievements

The specific aim of our project was to develop a novel single-molecule ultramicroscope capable of controlling protein conformations and analyzing protein activities in real time. Our project has been focused on two primary tasks: (1) to develop and demonstrate correlated single-molecule FRET measurements and AFM single-molecule force-pulling manipulation (AFM-FRET) of the conformations of enzyme proteins; and (2) to demonstrate a 10:1 range of controlled enzyme reactivity, substrate-enzyme binding affinity, and substrate selectivity by using AFM-FRET single-molecule conformational manipulation approaches. We have made significant advancements on achieving these goals.

(1) Developed and set up a combined AFM-FRET microscope

Combining the state-of-the-art atomic force microscopy and single-molecule fluorescence resonant energy transfer microscopy, we have developed a novel AFM-FRET ultramicroscope that is capable of control protein conformations for creating new protein activity, affinity, and

selectivity. This new technique serves a milestone of next-generation single-molecule spectroscopy and its application on exploring unprecedented soft material properties.

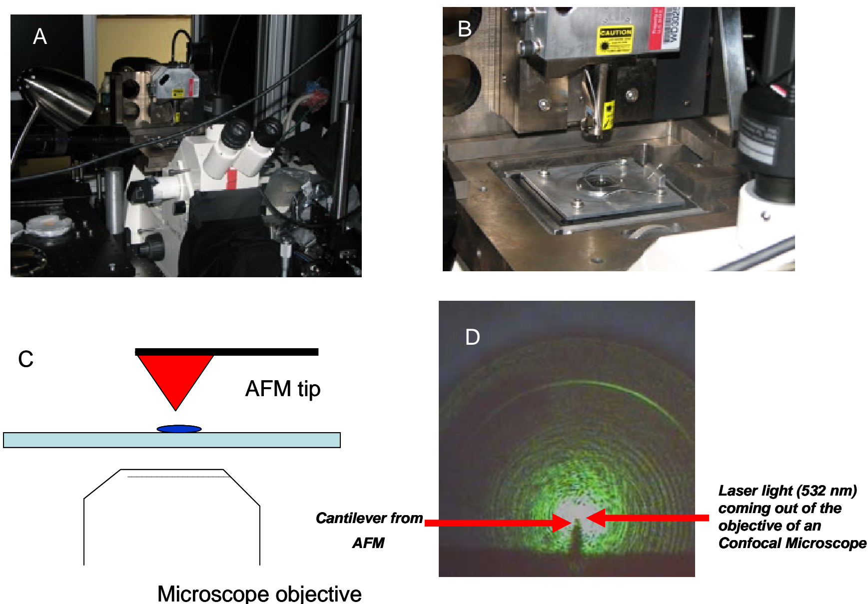

Figure 1: A home-modified AFM-FRET single-molecule imaging and spectroscopic microscope. AFM module and single-molecule fluorescence inverted microscope are positioned in an over-and-under configuration. **(A)** Overview of the single-molecule AFM-FRET ultramicroscope. **(B)** A close-up of the AFM module and the optical microscope focal point where the measurement is conducted on a surface-modified glass cover slip. **(C)** Conceptual diagram of the correlated microscope. **(D)** An overview of the AFM tip cantilever from an inverted single-molecule FRET imaging microscope, and the AFM tip is coaxial with the microscope laser illumination focal point. The image was taken from our inverted microscope: we look up from the microscope objective and see the laser reflection from the tip and the AFM cantilever.

(2) Demonstrated controlling AFM tip position in a sub-nanometer precision

A single-molecule protein can be held and be subjected to force-pulling under the AFM tip. Our single-molecule AFM-FRET imaging microscope can also shake the single-molecule protein at a given frequency from 0.1 Hz to 500 KHz. It has been predicted from physical theory that perturbing an enzyme protein with an oscillation force comparable to the enzymatic reaction turnover frequency, enhances protein enzymatic reaction activity by as large as 100 folds. We intend to demonstrate this fundamental theoretical prediction that can only be demonstrated by single-molecule experiments.

Control of Protein Conformations

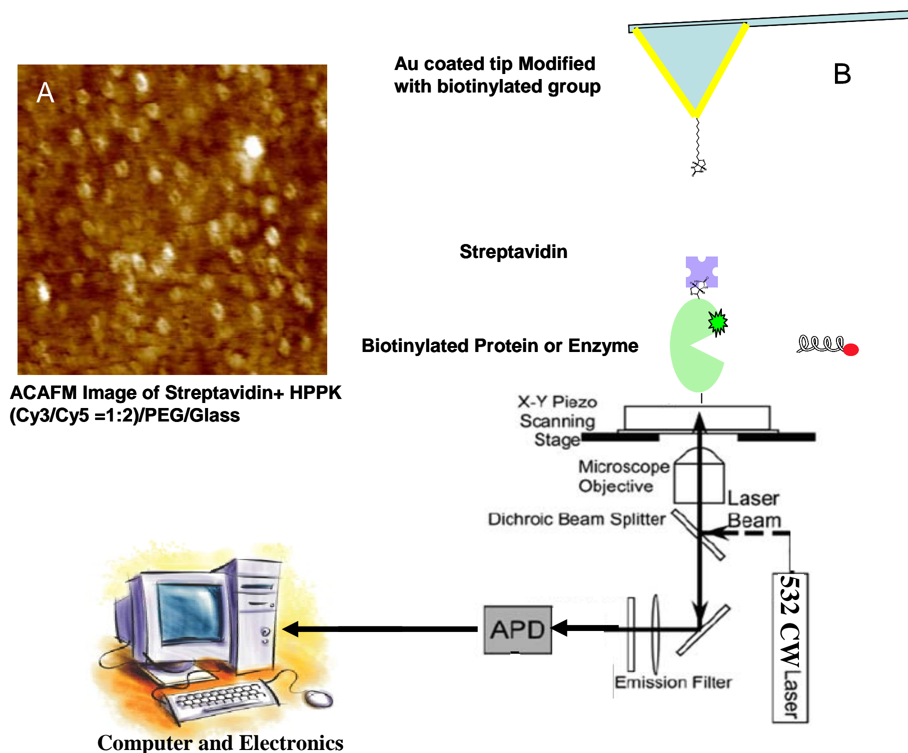

Figure 2: **(A)** An experimental (AC-mode) AFM image of single-molecule HPPK enzyme proteins tethered on a glass surface. The AFM first locates the proteins and then "hooks" on the individual proteins by biotin-streptavidin linkers using a biotinylated AFM tip. The HPPK proteins are labeled with FRET donor-acceptor dye pairs, and protein conformational changes introduced by the AFM tip force pulling are to be probed by single-molecule FRET. The glass surface is covered with biological compatative poly-ethelyne glycole (PEG) with 1 out of 10,000 biotinylated PEG to link to proteins. **(B)** A conceptual drawing of the AFM-FRET single-molecule ultramicroscope. There are three major components: The AFM microscope on the top is the “hand” to manipulate and control single-molecule protein conformations; the single-molecule FRET detection system is the “eye” to probe the conformational changes and the protein activity changes; and the laser excitation system to “light up” the single-molecule protein.

(3) Achieved AFM-force pulling single-molecule kinase enzyme (HPPK) proteins

We have demonstrated an AFM controlling protein conformations for HPPK enzyme proteins using our AFM-FRET ultramicroscope. Close-loop AFM scanning module can be position at a given x-y-z position and held on the position. The z-position change increment can be as little as 2 Å. The AFM module can also change the z-position continuously when conducting a single-molecule force pulling to fold or unfold a protein molecule. The single-molecule fluorescence

imaging microscope has a multi-channel detection with multiple APD detectors for FRET donor and acceptor fluorescence detections. Laser excitation wavelength can be selected by using different lasers and optics. Figure 3 and Figure 4 show the control of the conformations of HPPK enzyme proteins and calmodulin (CaM) signal proteins by using single-molecule AFM force pulling.

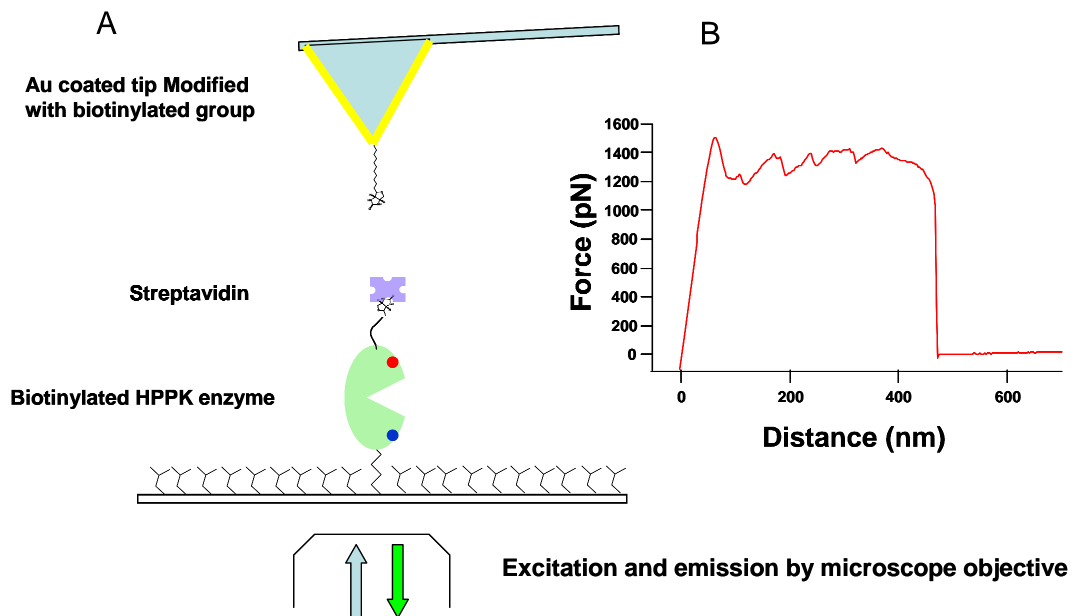

Figure 3: Single-molecule AFM force pulling a HPPK enzyme molecule using combined AFM-FRET imaging microscope. **(A)** A simplified sketch of the instrumentation. The biotinylated and Cy3-Cy5 labeled HPPK enzyme is tethered to polyethylene glycol covered glass cover slip at low density. Streptavidin is introduced to bind the biotinylated HPPK, and the excess streptavidin will be washed away before an experiment. The cover slip is mounted on a closed loop x-y electropiezo scanning stage controlled by computer. AFM tip from the top will be positioned at the coaxial position of the laser focus. In a force pulling experiment, the AFM tip is biotinylated. **(B)** An experimental raw data of the single-molecule force pulling curve of a HPPK enzyme protein, which is the first of its kind data. We have obtained repeatable force curves for this system, and one of the tasks of our project is to use AFT-FRET correlated measurement to specifically identify the conformational origin of each saw-teeth shaped force response. The actual pulling distance (x-axis) will be factor of 10 smaller for the proteins after a deconvolution fitting to remove the cantilever bending motion.

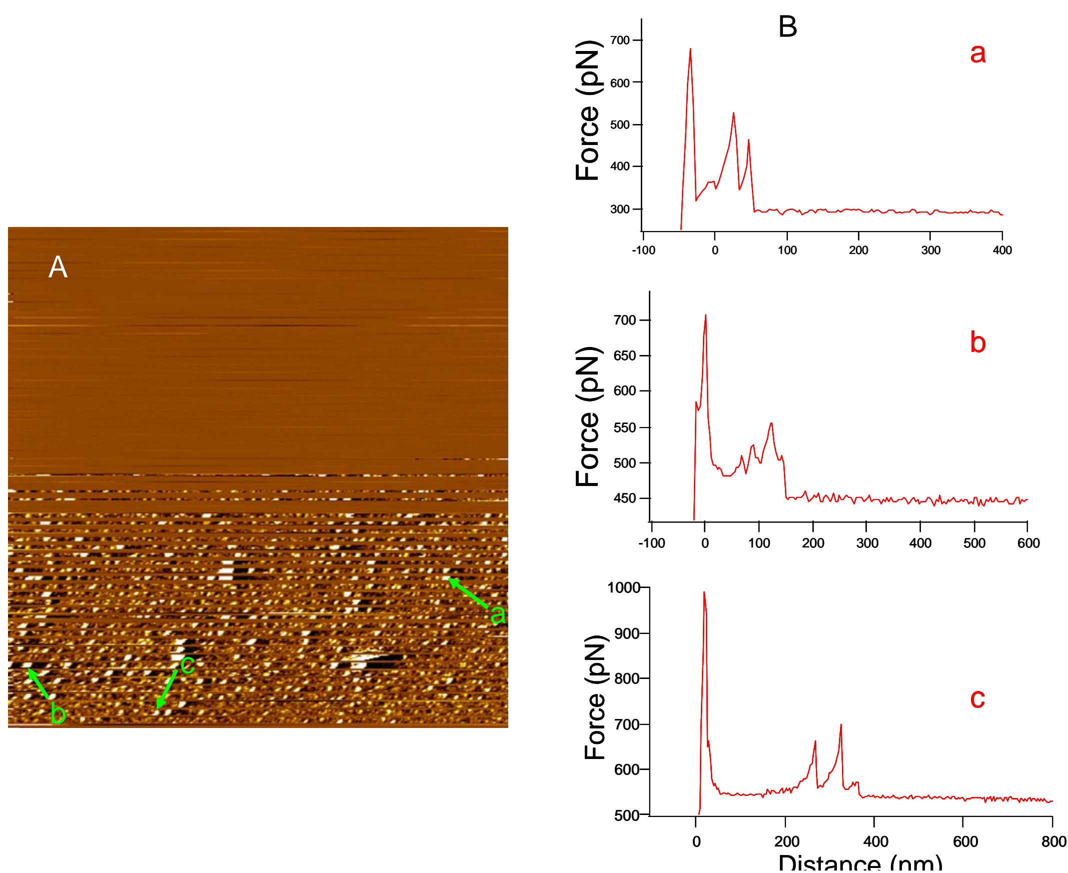

Figure 4. 2D force mapping of proteins on a surface using scanning AFM force pulling of AFM-FRET ultramicroscope. The biotinylated calmodulin (CaM) proteins was first identified in a 2D force screening imaging with a force pulling recording at each pixel scanned, and then the conformations of the CaM proteins were perturbed and controlled by AFM force manipulation. **(A)** AFM force volume experiment using biotinylated AFM tip to map a surface tethered with PEG + S-S Linked biotinylated CaM/Streptavidin in PBS buffer solution (pH 7.4). The image dimension is 10μmX10μm. Single-molecule AFM force pulling a HPPK enzyme molecule using combined AFM-FRET imaging microscope. AFM tip from the top will be positioned at the coaxial position of the laser focus. **(B)** Typical experimental raw data (a, b, and c) of the single-molecule force pulling curve of single-molecule CaM proteins. The actual pulling distance (x-axis) will be factor of 10 smaller for the proteins after a deconvolution fitting to remove the cantilever bending motion.

(4) Demonstrated single-molecule FRET probing HPPK enzymatic reaction activity

We have demonstrated a detailed study of enzymatic activity measurement for HPPK enzyme proteins using our AFM-FRET ultramicroscope (Figure 5A and 5B). The enzyme, 6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK), is a 35KDa monomeric protein.

HPPK catalyzes the pyrophosphorylation reaction leading the conversion of 6-Hydroxymethyl-7,8-dihydropterin (HP) to 6-Hydroxymethyl-7,8-dihydropterin pyrophosphate (HPPP) in the presence of ATP during the first step of the folate biosynthesis pathway. There are three loops present in HPPK, loop 3 undergoes the most dramatic conformational changes in each catalytic cycle. The substrates (HP and ATP) induce the opening and closing of loop 3 thereby controlling the enzymatic turnovers. Probing one molecule at a time provides us with the information on distributions and time trajectories of observables that otherwise remain hidden in the veil of ensemble measurements. We labeled the enzyme HPPK with Cy3 (donor) and Cy5 (acceptor) dye molecules as shown in Figure 5C. Herein, using single molecule fluorescence resonance energy transfer (smFRET) we revealed how the substrate induced conformational changes in HPPK regulates the enzymatic reaction dynamics (Figure 6).

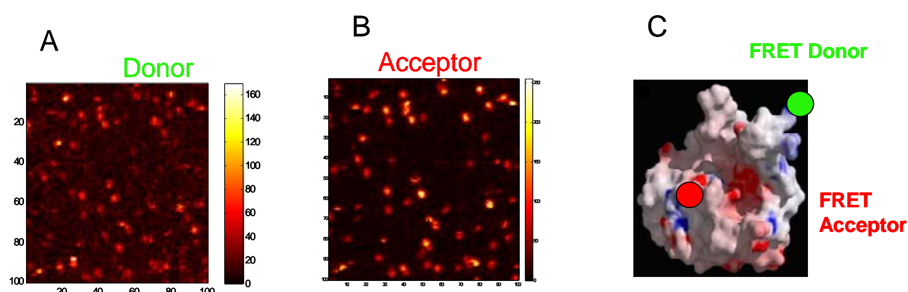

Figure 5. (A and B) Single-molecule FRET imaging of Cy3-Cy5 labeled HPPK proteins. We were able to record the single-molecule enzymatic reaction turnovers by probing the FRET change with the conformational motion during each enzymatic reaction turnover cycle. The enzyme protein conformation can be controlled and manipulated by an AFM tip of the single-molecule AFM-FRET microscope. **(B)** Apo-HPPK crystal structure with Cy3-Cy5 FRET labeling marked, showing Cy3 on loop 3 and Cy5 on core of the HPPK protein.

Single-Molecule FRET Probing Enzymatic Conformational Dynamics

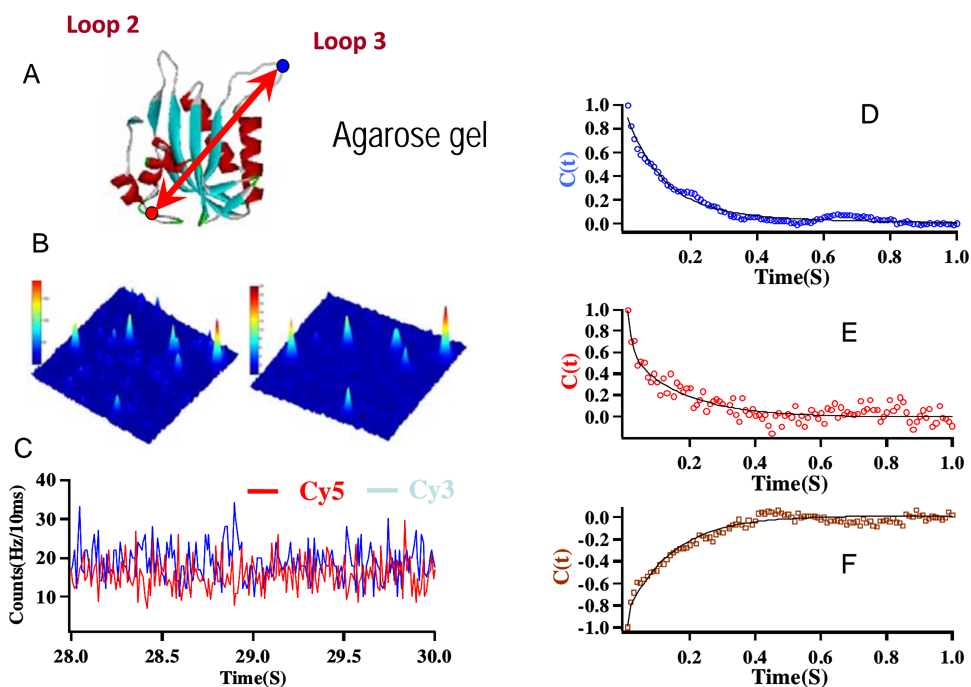

Figure 6: Single-molecule enzymatic reaction activity shows a broad range of variations (10:0.5) that can be measured and the reaction rate can be analyzed by the single-molecule AFM-FRET microscope. **(A) and (B),** Fluorescence images (10μm ×10μm) of single HPPK molecules labeled by Cy3 (Fig 2a) and Cy5 (Fig 2b) in the presence of 200 μM ATP and 200 μM HP. The emission is from the fluorescent active site, amino acid 88 (labeled by Cy3) and amino acid 142 (labeled by Cy5). The image was taken with an inverted fluorescence microscope (Axiovert-200, Zeiss) by raster-scanning the sample with a CW focused laser beam of ~1mW at 532 nm. Each individual peak is attributed to a single HPPK molecule and the intensity variation between the molecules is due to the different longitudinal positions in the light depths. **(C)** A part of the Intensity-Time traces of the donor (Cy3, blue) and acceptor (Cy5, red) labeled HPPK in the presence of 200 μM ATP and 200 μM HP. The anti-correlated nature of the motions of loop3 and the reactive center is clearly visible. **(D) (E) and (F)** Normalized Correlation functions, *(C(t))* of the donor (blue, 2C) and the acceptor (red, 2D) (auto-correlation) and donor-acceptor (brown, 2F) (cross-correlation) deduced from the single molecule Intensity-Time trajectories in 2B.

Conclusion

We have achieved the primary goal of our project: developed and demonstrated a novel single-molecule experimental technique capable of controlling protein conformations and imaging chemical or biological processes with both nanoscale spatial resolution and single-molecule FRET spectroscopy. Our project has provided a new stage in characterizing and analyzing the relationship between protein structure and function, which serves as an ultimate soft material

manipulation and analysis technique for exploring unprecedented material properties. The further development of this new stage in the next phase will feature rapid and sensitive analyses of protein structures and functions.

Recent related publications

1. Xuefei Wang, and H. Peter Lu, "2D Regional Correlation Analysis of Single-Molecule Time Trajectories," *J. Phys. Chem. B.* **112**, 1492--14926 (2008).

2. Duohai Pan, Dehong Hu, Ruchuan Liu, Xiaohua Zeng, Samuel Kaplan, H. Peter Lu, "Fluctuating Two-State Light Harvesting in a Photosynthetic Membrane," *J. Phys. Chem. C*, **111**, 8948-8956 (2007).

3. Qiang Lu, H. Peter Lu, and Jin Wang, "Exploring the Mechanism of Flexible Biomolecular Recognition with Single Molecule Dynamics," *Phys. Rev. Lett*. **98**, 128105 (2007).

4. V. Biju, D. Pan, Yuri A. Gorby, Jim Fredrickson, J. Mclean, D. Saffarini and H. Peter Lu , "Correlated Spectroscopic and Topographic Characterization of Nanoscale Domains and Their Distributions of a Redox Protein on Bacterial Cell Surfaces," *Langmuir* **23**, 1333-1338 (2007).

5. Ruchuan Liu, Dehong Hu, Xin Tan, and H. Peter Lu, "Revealing Two-State Protein-Protein Interactions of Calmodulin by Single-Molecule Spectroscopy," *J. Am. Chem. Soc.* **128**, 10034-10042 (2006).