LOCAL NEWS:

During the past year a number of users had the opportunity to address and extend their research with the resources available at the RLBL. The special character of this laboratory could be best described as the ability to perform all sorts of useful optical experiments and in sharing experience with other people. All of us, the staff, post-doctoral fellows and graduate students involved in this resource, are always willing to help out with, to improve, and to newly design experimental setups in order to meet the needs of our users.

In this newsletter we would like to concentrate especially on the ever expanding field of single molecule spectroscopy and its application for biophysical and biomedical research. Optical studies of single molecules became feasible through a combination of efficient collection and detection of fluorescent light coupled with a careful minimization of background signals. Compared to classical spectroscopic experiments, one of the main advantages of single molecule spectroscopy is the possibility to obtain experimental information of static and dynamic inhomogeneity in the system under study. The observation of such stochastic events allows for a construction of a frequency histogram of the distribution of values for the experimental parameter, which contains more information than an averaged value alone.

Within the last couple years we have built and extended two instruments for single molecule spectroscopy, which form now a major part of our service capabilities for outside users. Dr. Erwen Mei, who is the staff person responsible for this aspect of our resource, would like to take the opportunity in this newsletter and inform you more extensively about our capabilities. Please see his contribution in the following article.

Following Dr. Mei’s contribution we report on a research article about the single molecule studies of a GCN-4 protein and its dynamics in our feature article as an example of a successful collaborative research project applying single molecule techniques.

A principal focus of our core research program remains the development and application of two-dimensional IR spectroscopy, which are infrared analogues of NMR. This subject formed the topic of the feature article in the last newsletter. In order to keep you informed about further developments and new results about these activities of the research resource, we include once more our list of recently published research articles following the feature article.

I also would like to mention our homepage on the World Wide Web (rlbl.chem.upenn.edu) which gives a brief summary of all our research interests and activities. Any prospective user of our facility may find useful first information there. If you have any comments or any thought of becoming a user of the RLBL, please send an email to my attention.

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Single Molecule Studies at RLBL

Currently, we have two two-channel confocal microscopes equipped with different laser sources (Ar-Ion lasers, Ti:Sapphire oscillator, ml-Nd:YAG laser, ps dye lasers), which enable us to do a great variety of single molecule and single cell studies.

Single molecule detection coupled with time correlated single photon counting technology can offer us a wealth of information not available from bulk measurements. This method has been applied to investigate the conformation change of single tRNA\textsuperscript{Phe} molecules under physiological conditions. By measuring the lifetime of single tRNA-TMR adduct, the conformation change of single tRNA\textsuperscript{Phe} molecules has been probed, evidenced by the distribution of lifetimes.

The fluorescence resonance energy transfer (FRET) is a technique, which has been widely used in probing the distances between sites on multi-subunit proteins, and the thickness of biological membranes. Usually, proteins and other biological assemblies exhibit microscopic structural heterogeneity, so the single molecule-FRET technique is of particular interest for these studies.

The single molecule FRET method was used at RLBL to study the folding and unfolding dynamics of single GCN4 peptides in physiological solution. By monitoring the transient fluorescence intensities simultaneously with a two-channel confocal microscope, the folding and unfolding process and the energy transfer efficiency has been investigated at the single molecule level, thereby studying the conformational change of a single biomolecules under physiological conditions. Another single molecule FRET method has been developed at RLBL for investigating energy transfer processes of a donor-acceptor system in a rigid medium. In this method, TMR molecules are used as donor and immobilized in a rigid polymer film. By measuring the lifetime of single TMRs, we can study the process of FRET at a single molecule level to determine distance and angular distributions.

Another single molecule detection technique that has been extensively explored at RLBL is to probe the orientational motion of single molecules. Two methods have been developed. In one method, an electro-optic modulator is used to modulate the polarization of the excitation light. Recording the fluorescence transient intensity of single molecules, we can obtain orientational information of individual molecules. This method has been used to study the dynamics of structural deformations of immobilized single light-harvesting complexes. In another method, a two channel confocal microscope has been developed for probing the rotational and translational diffusion of single molecules. In this case, circularly polarized light is used to excite dye molecules and the fluorescence intensity is recorded at two orthogonal polarization. This method has been used in a collaborative project with Jane Vanderkooi’s group to investigate the rotational and translational diffusion of single protein molecules in the cryoprotector trehalose.

Developing new imaging and spectroscopy techniques for investigating chemical and physical properties of biomacromolecules in living cells has also been a major direction pursued at RLBL. Single photon and/or multiphoton fluorescence spectroscopy has been coupled to a confocal microscope for
investigating the conformational changes of the interferon-gamma receptor and interleukin-5 receptor complex on the membrane of living cells. Again, a FRET method is used here.

Please feel free to contact me for any further question about using the single molecule spectroscopy equipment at the RLBL.

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FEATURE ARTICLE

Equilibrium Dynamics of single 2-stranded coiled-coil peptides studied by fluorescence resonance energy transfer.

Reported by Robin M.Hochstrasser and David S.Talaga

Introduction

The RLBL has established a single molecule microscopy laboratory that can accomplish a wide range of experiments using fluorescence techniques. With the object summarizing the experimental capabilities of this lab I will describe some recent work done through a collaborative project in the Resource. Many of the commonly occurring practical and interpretive issues in single molecule work were brought out by this study of a small coiled-coil.

However, the RLBL has ongoing collaborations in measuring single molecule responses of proteins, nucleic acids and even in living single cells. Anyone who is interested in learning more about the single molecule capabilities of the Resource should contact us through the addresses given below.

Equilibrium Dynamics of Coiled-Coils: Late last year we reported (1) single molecule measurements on the folding and unfolding conformational equilibrium distributions and dynamics of a disulfide crosslinked version of the two-stranded coiled coil from GCN4. The present article is an embellishment of that report, with an emphasis on the single molecule experimental and interpretive methods used in the RLBL. The original paper containing all the essential scientific ideas expressed here, was coauthored by Wai Leung Lau and William F. DeGrado of the Department of Biochemistry and Biophysics, Heinrich Roder of the Fox Chase Institute for Cancer Research and David S. Talaga, Jianyong Tang, Yiwei Jia and me from the RLBL. The peptide was arranged to have a fluorescent donor and acceptor at the N-termini of its two chains, and a Cys disulfide near its C-terminus. Folding was expected to bring the N-termini of the two chains closer together resulting in an enhancement of fluorescent resonant energy transfer (FRET). Indeed, single-molecule folding studies provided novel information concerning the distributions of conformational states in folded, unfolded, and dynamically interconverting states.

Fluorescence Confocal Microscopy at RLBL: The inverted scanning confocal microscope at RLBL
has been designed for flexibility and the ability to carry out a number of different experiments simultaneously. Single molecules can be excited over a wide range of wavelengths in the visible and near UV or by two-photon absorption. In the present example they were excited by circularly polarized light from a 76MHz mode-locked Nd:YAG laser, frequency doubled to 532 nm. This means of excitation eliminated the effects of azimuthal angular fluctuations of the transition dipoles on the absorption probabilities of the single molecules.

The microscope incorporates multichannels for separate detection of different wavelengths and polarizations of quantum yield detection of single photons at the donor and acceptor fluorescence wavelengths. Fluorescence images and fluorescence spectra can be recorded on the RLBL instrument as can fluorescence correlation spectra and lifetimes using single photon counting methods. In correlation spectroscopy the intensity vs. time signals are measured when the single molecules are diffusing through the focused laser beam. The single molecule fluorescence experiments were carried out in the RLBL by Yiwei Jia, David Talaga and Jianyong Tang using this type of instrumentation, illustrated in Figure 1.

The GCN4-Pf Peptide: The peptide that was used in our work was designed in the laboratory of W. F. DeGrado and synthesized by Wai Leung Lau in the Department of Biochemistry and 

![Figure 1: Single molecule fluorescence apparatus.](image-url)
Biophysics at Penn. It contains a short stretch of a two-stranded coiled coil from the yeast transcription factor, GCN4 (2-4), as cartooned in Figure 2, a disulfide cross-link near its C-terminus to provide an intramolecular folding situation, and a poly-Glu tag to aid in immobilizing the peptide onto a positively charged aminopropyl-silanized cover slip. To allow FRET measurements, the peptide included a fluorescent donor (5-carboxy-rhodamine 6G) and an acceptor (Texas Red). The variant employed in this study, designated GCN4-Pf, is a disulfide-crosslinked heterodimer, whose individual chains differ only in the nature of the fluorescent probes.

**Single Molecule Experiments:** A typical time recording of the fluorescence of an immobilized molecule of GCN4-Pf is shown in Figure 3. The emitted photons were monitored at the wavelengths of the donor and acceptor fluorescence. As the distance between fluorophores increases or the angles between the transition dipoles becomes less favorable, the fluorescence of the donor increases relative to that of the acceptor. The observed anticorrelation of the donor and acceptor signals indicates that the fluctuation amplitude in the trajectories is influenced by changes in the donor/acceptor couplings and the orientations of the transition dipoles of the dyes relative to the surface normal (see below). These time records were collected for a given interval and then converted into time records of quantum yields for energy transfer ($\Phi_{ET}$). The application of Forster energy transfer theory with certain assumptions permits these efficiencies to be converted into distances. As expected there was an increased average value of

![Figure 2: Schematic representation of the folded and an unfolded structure of GCN4-Pf. The negatively charged glutamic acids at the C terminus of the peptide provide adhesion to the surface.](image)
the donor/acceptor distance in the unfolded state.

Figure 3: Typical time resolved signal records for the donor (white line) and acceptor (black line) fluorescence channels from a single GCN4-Pf at pH 6.1.

In an earlier paper (5), published as a contribution to a Special Issue on Single Molecule Spectroscopy in Chemical Physics, we had examined the distribution of donor/acceptor distances under conditions where the peptide (in solution) was known to be nearly fully folded (0 M urea), unfolded (7.4 M urea), or in a dynamic exchange between folded and unfolded states. As the concentration of urea increased, we found that the mean end-to-end distance computed from application of Förster theory also increased, and the distributions broadened. Different distributions were obtained when the trajectories were averaged over different time intervals, implying that the peptide was exhibiting dynamic behavior under all three conditions.

Fluorescence Correlation Spectroscopy: The proximity of a surface influences the dynamics of the folded and unfolded states of an immobilized molecule. In order to evaluate the contribution from surface related processes parallel experiments on GCN4-Pf in bulk solution were carried out using fluorescence correlation methods which directly measure the conformational and folding dynamics free from surface effects. The inter-chromophore distances obtained from the energy transfer efficiencies exhibit a distribution characteristic of the particular conformational ensemble of the peptide. Each peptide fluctuates within this distribution with characteristic times that depend on the details of the free energy landscape that the peptide explores during the observation time. Figure 4 shows the donor/acceptor distance distributions for GCN4-Pf in solution and on the surface, computed from the values of $\Phi_{ET}$ using Förster theory. The solid lines represent simulations of the widths of the distributions expected from the shot noise alone. The observed distributions were found to be significantly broader than the shot noise.

Auto and Cross Time Correlation Functions of the Energy Transfer: Conformational fluctuations of the protein cause changes in the donor/acceptor relative orientations and separations. The resulting fluctuations in the single molecule signals can be used to obtain the donor...
Figure 4: Distributions of the average donor/acceptor distances for immobilized (left) and solutions (right) of GCN4-Pf.

and acceptor auto and cross time correlation functions. The averaged time correlation functions (Figure 5) show multiple time scales in each of the denatured conditions with the decay rates varying from molecule to molecule. If energy transfer were the only mechanism of intensity modulation the amplitude of the negative cross time correlation would be the geometric mean of the autocorrelation amplitudes. However the correlation function was found to be negative but with a magnitude at time zero less than the geometric mean of the variances of the autocorrelation functions. Therefore, energy transfer must not be the only mechanism for modulating the signal. Fluctuations in the angle between the transition dipoles and the axis of the microscope increase the magnitude of the time correlation functions. Correlated fluctuations of this type make a positive contribution to the cross correlation, effectively canceling some of the negative cross correlation. Even in the presence of this effect it is clear from the data that the signals are exhibiting dynamic modulation of the energy transfer distance.

If the protein were to fluctuate between folded and unfolded states we expected that the time correlation functions would exhibit an exponential decay with a time constant equal to the reciprocal of the sum of the folding and unfolding rates. The maximum amplitude of the correlation function, corresponding to the maximum variance of the fluctuations, was expected to occur when the populations of folded and unfolded states are equal. The correlation functions we measured, however, exhibited nonexponential decays. Nevertheless, the contribution to the decay of the time correlation functions that was most sensitive to the concentration of urea was in reasonable correspondence with the kinetics measured by bulk relaxation methods in the laboratory of Heinrich Roder at the Fox Chase Cancer Center.

Effective Diffusion Properties of the Configurations: Each value of the effective donor acceptor coordinate corresponds to many configurations. Thus, we realized it would be valuable to examine the distribution function of some other variable at each coordinate and that the calculation of a distance trajectory should allow additional information to be extracted from single molecule measurements. A given donor/acceptor distance can correspond to either a folded or unfolded configuration, but folded configurations should have very different fluctuations than unfolded ones. An
important advance was made by David Talaga who evaluated the first derivatives of the distance trajectory with respect to time, thereby yielding an instantaneous velocity which allowed the extraction of an effective “diffusion” factor, whose value at each donor/acceptor distance depended on the solution conditions. The result that this factor is smallest for 7M urea confirmed the idea that interchange between unfolded configurations of GCN4-Pf on the silanized glass surface is the slowest of all the denaturant conditions.

Figure 5: Time correlation functions and their dependence on urea concentration for immobilized GCN4-Pf. A=acceptor, D=donor time autocorrelation, C=cross time correlation function.

**The Effects of Immobilization:** In all our single molecule experiments at the RLBL we question the effects of surfaces on the properties of immobilized molecules. Yet immobilization is a key, often a necessary situation for single molecule work. One of the objectives of our investigation of this peptide was to compare distributions of $\Phi_{ET}$ for surface-adsorbed GCN4-Pf with those of the same peptide in solution. Previously we had reported a broad feature in the 7M urea distributions at relatively low $\Phi_{ET}$ and attributed it to portions of the trajectory where the unfolded peptide has its conformational fluctuations slowed due to interactions with the surface (2). This conclusion was confirmed by the present study. The broad feature was indeed absent in the distributions obtained from freely diffusing molecules (Figure 4). The slowest time scale in the correlation functions was independent of urea concentration and also could be ascribed to surface related processes. Otherwise the distributions observed on the surface and for molecules freely diffusing in solution were quite similar in width and position leading us to conclude that the main peaks are representative of the fluctuating peptide with minimal contributions from surface interactions. These are issues that need to be confronted in all single molecule experiments. The evidence from the two types of distribution measurements and the correlation functions indicates in the present case that there are dynamic fluctuations in $\Phi_{ET}$ that persist for times greater than 1.5ms that are independent of the surface.

**The Orientational Averaging in the Case of Single Molecules:** At issue in all FRET experiments and a continuing focus of our experimental and theoretical efforts at RLBL is the determination of the
transition dipole angles in donor acceptor pairs. It is well known that this knowledge is needed to convert the energy transfer efficiency into a distance. For single molecule experiments there are three main considerations. The first is how adequately the molecule samples the available orientational distribution on the time scale of the measurement. This averaging often should be complete on the ns time scale. The second is how often the distribution is sampled by the measurement. Enough photons (greater than ca. 10) are sampled in conventional single molecule experiments to ensure that the an orientational distribution can be averaged with not unreasonable accuracy. This is easily verified from numerical simulations. The final issue is the proper magnitude of the mean value of the Forster orientation factor. Often this mean is set equal to its isotropically averaged value of 2/3. This is more likely to be appropriate for the unfolded distribution in which the dyes should reorient efficiently into a sufficiently large angular space in the time between observed photons. We at RLBL and many others have learned that simultaneous measurements of the fluorescence polarization and lifetimes of the donors and acceptors will greatly assist in the conversion of energy transfer efficiencies to a distance distribution.

**Folded and Unfolded State Distributions:** For a two state system such as GCN4-Pf, the folded state was expected to exhibit a narrow range of coordinates while the unfolded state distribution was expected to be broader. If the motional averaging within these distributions was sufficiently fast their experimentally determined widths would be determined by the shot noise. The observed distributions for the molecules in solution, free from surface interactions, turned out to be broader than shot noise indicating that the energy transfer measurement senses the presence of motions on time scales comparable with or slower than the observation window of 1.5 ms. The slowness of part of the conformational averaging of the folded state was surprising given the expected well defined structure. This prompted us to suggest there might be contributions to the distributions and to the time correlation functions from the slower conformational motions of the donor and acceptor probes at the N-termini, relative to the peptide. Future experiments with a time resolution around 1 ns would be expected to permit a firmer resolution of this and related questions that have important general implications.

**Summary**

The power of single molecule experiments is evident when we consider that we obtained an experimental measure of the trajectory of the folding and unfolding reactions as they occur repeatedly at equilibrium. Understanding how the empirical folding coordinate relates to actual distributions of structures is an important step in relating the observed distributions and correlation functions to protein folding. This work provided a direct analysis of the trajectories of a small model protein undergoing exchange between folded and unfolded states. The data are consistent with the bulk macroscopic data, but provide additional information on the end-to-end distance distributions, potentials of mean force and the diffusion coefficients.

The reader is referred to the original articles for further details of these
Acknowledgements: The work in references (1) and (2) was supported by GM54616 (to William F. DeGrado), GM12592 (to Robin M. Hochstrasser) and GM48130 (to William F. DeGrado and Robin M. Hochstrasser) with instrumentation developed under RR01348. David S. Talaga was supported by NIH NRSA F32-GM18589.

Literature:


SUMMARY OF CURRENT TECHNOLOGICAL DEVELOPMENT AND RESEARCH AT RLBL

The main subjects under investigation at RLBL are shown below. If your research may be interfaced with any of these approaches we urge you to contact us. A fuller description of each of these categories can also be found on our Web site at http://rlbl.chem.upenn.edu.

- **Dynamics of photoactivatable proteins and other biological structures**: Methods are being developed to examine the responses of biological systems to light by pump/probe and nonlinear spectroscopic methods encompassing spectral regimes from the UV to the far IR and covering femtosecond to second timescales. Techniques include: single and multiple wavelength transient spectroscopy (UV/Vis, vibrational IR, Terahertz), photon echoes, two photon absorption and time-correlated single photon counting.

- **Methodologies to investigate protein folding and macromolecular conformational dynamics**: Detection and characterization of intermediate states in conformational dynamics and unfolding is another developing technology at RLBL. Laser-based temperature-jump instruments are available for these investigations.

- **Investigations of single molecular assemblies using confocal and atomic force microscopes**: It is now possible to examine the properties of single molecules using fluorescence in association with confocal microscopy. The RLBL is coupling single molecule detection methods with mature time
correlated photon counting technology, polarization scanning and pulsed laser experiments.

- **Energy transfer and fluorescence monitoring of biological dynamics**: Monitoring fluorescence lifetimes and anisotropies reveals details of protein dynamics. Techniques are being developed at the RLBL to monitor these properties of fluorescing species on the femtosecond to nanosecond timescale.

- **Development of time resolved far-IR (terahertz) probes for protein dynamical changes**: New powerful sources of THz and far-IR radiation are developed and used as laboratory THz source.

- **Two-dimensional infrared spectroscopy and infrared analogues of NMR**: Heterodyned photon echo spectroscopy and spectrally resolved three pulse IR photon echoes are employed to investigate the amide I region and other transitions of peptides and small proteins.

### A SELECTION OF RECENT COLLABORATIVE PUBLICATIONS


### A SELECTION OF RECENT CORE PUBLICATIONS


Application for Use of the RLBL

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Abstract: Describe briefly (200-250 words) the scientific goals and methods.

Logistics: Equipment to be supplied by applicant, needed from RLBL, and anticipated time.

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