and thoroughly washed to remove any non-internalized fluorescence. Internalized molecules can be tracked at the single-molecule level, and both single-cell and single-molecule FRET can be measured.
We have explored the effect of electroporation voltage on the internalization efficiency of short DNA fragments and proteins, and have found a linear relationship. Hence, an appropriate voltage can be selected depending on the application of interest, such as whether single-molecule or ensemble measurements are desired. In addition, we have optimized the buffer and salt conditions for electroporation, in terms of maximizing internalization efficiency whilst preserving protein integrity.
Various conditions have been tested for cell washing, including the use of salt and detergent in the washing buffers. The medium used for cell recovery after electroporation has also been noted to affect the efficiency of cell washing. A significant improvement in the removal of non-internalized fluorescence has been achieved by cell filtration. In the case of proteins prone to aggregation, cell filtration has also been found to remove any high-molecular weight species from the cell suspension.
Finally, we have found free dye to be internalized at much higher efficiency than the labeled biomolecules, and hence it is important to remove any contaminating free dye from the samples used for electroporation. We have optimized the methodology for determining and minimizing the amount of free dye in our samples, and have obtained samples that show less than 1% free dye contamination, which is at the level of background autofluorescence.

2319-Pos Board B11
Photo-Activated Crosslinking Mass Spectrometry for Studying Biomolecular Interactions
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The structural and mechanistic analysis of biomolecular interaction is important for understanding the molecular basis of a wide range of biological phenomenon. Relatively weak interactions between functional molecules may play crucial roles in regulating highly networked and dynamically controlled biological systems. This type of interaction, however, is more challenging to study because of its transient nature. As a general solution to the problem, we propose the use of chemical crosslinking in combination with mass spectrometry and apply it to protein-protein interactions. Ultraviolet-activatable crosslinker molecules that are incorporated to the protein of interest can be utilized to capture transient interactions under a physiological condition by forming covalent bonds between interacting molecules in close proximity via carbene chemistry. The crosslinked sample is then enzymatically digested and analyzed by liquid chromatography-mass spectrometry. High mass resolution analysis ensures identification of crosslinked peptide species and enables spatial mapping onto available structural models obtained from crystallography or NMR spectroscopy. The proposed methodology is demonstrated with a model system of cytochrome c and its oxidase, where we find multiple binding modes and explore their possible role in controlling enzymatic activity.

2320-Pos Board B12
Protein Resilience and Fluorescent Protein Resistance to Photobleaching
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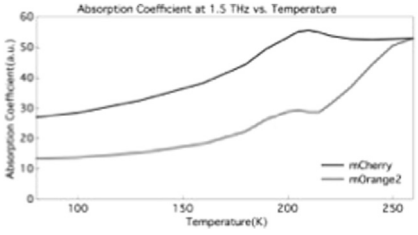
Fluorescent proteins (FPs) are ubiquitous in biophysics. To simultaneously tag many different biomolecules and allow excitation at lower wavelengths, FP's have been developed with excitation and fluorescence at wavelengths > 550 nm, however these red fluorescent proteins are more susceptible to photobleaching [1].



A possible reason for this is a decrease in the structural stability of the beta barrel, but X-ray B-factor measurements do not indicate this stability change. Zaccai introduced a measure of protein stability called resiliency [2], derived from the temperature dependent atomic mean squared displacement measured by neutron scattering. These facility-based measurements require ~100 mg of protein, so are not conducive to systematic testing of changes in resiliency with mutation. However table top THz optical absorption measurements have shown the same temperature dependence as [3]. Using this method we find the resiliency of mCherry is 2.5 times higher than mOrange2 consistent with mCherry's higher stability and longer irreversible photobleaching time constant (3.46 s) compared to mOrange (0.26 s).
[1] K. M. Dean et al., Biophys. J. 101, 961 (2011).
[2] G. Zaccai, Science 288, 1604 (2000).
[3] Y. He et al., Phys. Rev. Lett. 101, 178103 (2008).

2321-Pos Board B13
Microsecond Conformational Dynamics of Cytochrome C Revealed by Two-Dimentional Fluorescence Lifetime Correlation Spectroscopy
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Characterization of the folding process is a long-standing central issue in protein science. Single-molecule spectroscopy, especially that in combination with fluorescence resonance energy transfer (FRET), has been utilized as a powerful tool to explore the conformational heterogeneity of proteins and its transition dynamics on the sub-millisecond to second timescales. However, observation of the dynamics on the microsecond timescale is still challenging. Elucidation of protein dynamics in the microsecond region is very crucial to understand elementary processes of not only folding but also various biological functions of proteins. We recently developed a new single-molecule technique to quantitatively examine the microsecond dynamics of biomolecules based on fluorescence lifetime correlation analysis. This method, two-dimensional fluorescence lifetime correlation spectroscopy (2D FLCS) [1,2], was applied to the spontaneous conformational transition of cytochrome c (cyt c) in this study. One fluorophore, Alexa546, was covalently attached as a FRET donor to the single free cysteine residue of cyt c located in the C-terminal region. The temporal change in the donor fluorescence lifetime due to FRET between the donor and heme was then analyzed to evaluate the conformational transition dynamics of cyt c. We show that 2D FLCS reveals diverse conformers of cyt c and provides unambiguous information about their microsecond transition dynamics. This work demonstrates the high capability of 2D FLCS to elucidate the complex conformational transition dynamics of proteins.
References
[1] Ishii and Tahara 2013. J. Phys.Chem. B. DOI: 10.1021/jp406861u.
[2] Ishii and Tahara 2013. J. Phys.Chem. B. DOI: 10.1021/jp406864e.

2322-Pos Board B14
Measuring Protein Structural Heterogeneity with Two-Dimensional Infrared Spectroscopy
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Protein structure and heterogeneity is particularly difficult to measure due to lack of experimental techniques that combine structural sensitivity and sub-microsecond time resolution. Two dimensional spectroscopy is a new optical technique that measures protein structure and dynamics with ultrafast time resolution. The delocalized backbone C=O (Amide-I) vibrations reflect the global secondary structure of the protein. A 13C=18O isotope label on a residue red-shifts its frequency by ~60 cm-1, isolating the site from the main amide band. The label provides a unique spectroscopic handle on the structure (distances), heterogeneity, and hydrogen-bonding environment (solvent exposure) of the labeled residues, and the ultrafast time resolution is able to distinguish between different fast-exchanging conformational states.
We apply this new method to NTL9, a 39-residue α/β mini protein, by isotope labeling five different sites, including a dual-label across a type-I beta-turn. The structural interpretation is enabled by spectral simulations based on a recent Markov state model (MSM) built from millisecond-long molecular dynamics trajectories. Structures are assigned by matching the measured frequencies and lineshapes to simulated spectra for each Markov state. The excellent qualitative agreement between theory and experiment provides a solid set of structural constraints. We find a number of sub-states with different configurations, particularly in flexible regions of the protein, such as the type-I beta turn. Specifically, we find a significant population of bulged turn configurations. The results show that residues in the first and last turns of the helix exhibit multiple hydrogen-bonding environments reflecting the greater solvent-exposure within these regions of the backbone. Finally, the lineshapes serve to characterize the flexibility and stability of the backbone at the different sites. We find that β-strands remain relatively rigid whereas the turn and helix regions show increased flexibility, qualitatively matching b-factors extracted from crystallography.

2323-Pos Board B15
Conformational Equilibrium between the Sub States of the Acidic Denatured State of ACBP Determined by NMR Chemical Shifts and Metadynamics
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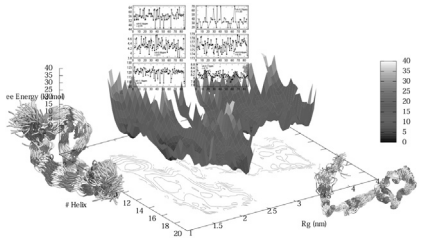
NMR Chemical shifts have recently gain renovated attention as a probe for both the structure and the dynamics of proteins. The structure and the dynamics of

folded proteins can be elucidated straightforwardly from the backbone chemical shifts at least for small to medium sized protein. In the denatured state and for intrinsically disordered proteins chemical shifts are routinely used as probe for secondary structure propensities and populations and have been also used as a probe for tertiary structure contact propensities. In this work we show how chemical shifts, together with an appropriate advanced sampling method, can be successfully used to determine the structure and the dynamics of the denatured state of ACBP, an 86 residues long proteins, in acidic conditions. Chemical shifts are used as replica-averaged restrained to improve the quality of the molecular mechanic force field in the Maximum Entropy framework and Bias-Exchange Metadynamics is used to achieve a converged sampling of the large conformational space of an unfolded protein.



2324-Pos Board B16

Mapping Proximity within Proteins using Fluorescence Spectroscopy: Tyr as Well as Trp can be used for Distance-Dependent Fluorescence Quenching Studies

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We have been developing a site-directed fluorescence labeling (SDFL) approach called TrIQ to assess distances within and between proteins, in a way complementary to traditional FRET methods. TrIQ, or Tryptophan-Induced Quenching, exploits the fact that some fluorophores are quenched by nearby Trp residues in a distance dependent fashion. Here we report two new advances to TrIQ, gleaned from recent studies of T4 lysozyme (T4L).

First, we find Tyr can also be used in TrIQ studies. Interestingly, Tyr has some key differences from Trp. Tyr has a smaller "sphere of quenching" for the fluorophore bimane, and only shows significant quenching for Cα-Cα distance of less than ~10Å, compared to ~15Å for Trp. Also, unlike Trp, Tyr cannot quench the fluorophore BODIPY.

Second, we find TrIQ can reliably assess the magnitude and energetics of protein movements, especially when a combination of Tyr and Trp are employed. We used TrIQ to measure a key movement in T4L by placing a bimane and Trp (or Tyr) on opposite ends of a ''hinge'' in T4L, sites predicted to be ~14.5Å apart (Cα-Cα distance) in the substrate bound state, and ~10.5Å in the empty state. The only substantial TrIQ was observed for the Trp sample in the empty state, and this quenching was abolished in a mutant (T26E) that covalently binds substrate. Tyr did not dramatically quench bimane in either the empty or bound conformation. Together, these results are consistent with the different distance constraints for quenching described above. Arrhenius analysis of the effect of hinge-bending movement on fluorophore lifetime suggests an activation energy on the order of 2-4 kcal/mol, and this value is not significantly affected by the ''active site mutation'' T26E.

2325-Pos Board B17

Exploring Structure and Dynamics of Human Aquaporin-1 by Solid-State NMR

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Membrane proteins are notoriously hard to study, especially in their native lipid environment. Solid-state NMR is a promising technique to study structure and dynamics of membrane proteins, and we have recently applied it to get insights into the structure, oligomerization, and conformational changes of a cyanobacterial photosensor (Wang et al., Nature Methods, 2013, in press). We now show that human aquaporin-1 (hAQP1), a water channel with multiple tissue localization, can be studied in its functional form with atomic resolution by solid-state NMR.

First, we developed a protocol for isotopic labeling of hAQP1 in methylotrophic yeast Pichia pastoris (Emami et al., J. Biomol. NMR, 2013, 55: 147-155). The functionality of yeast-expressed hAQP1 was verified by water transport experiments in proteoliposomes and specific inhibition by mercury. After optimization of lipid reconstitution by FTIR and NMR, we were able to obtain multidimensional solid-state NMR spectra of a superior resolution. Next, we were able to assign resonances for 188 out of 269 residues, including ~90% of the transmembrane region with the water pore and selectivity filters. From the determined chemical shifts, we derived secondary structure of the protein, which mostly agrees with the X-ray structure of the bovine homolog, but gives some additional insights into the interfacial regions. Finally, we conducted H/D exchange experiments, in which solvent accessibility and hydrogen bonding of the exchangeable backbone and sidechain atoms could be monitored. We found the evidence for hydrogen-bonding interactions between the longest extracellular loop and the selectivity filter. These results lay a solid foundation for future studies of interaction of hAQP1 with pharmacological blockers, suggested to be important in treatment against glaucoma, edema, cancer, and other diseases.

2326-Pos Board B18

Non-Thermal Induction of Conforamtional Rearrangment in Proteins by Far-Infrared Excitation

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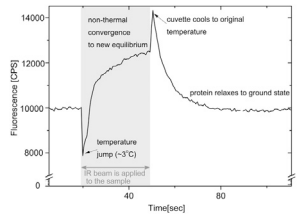
How does a protein respond to infrared excitation? Our aim was to induce a specific non-thermal conformational change of a protein by infrared laser excitation. The fluorescence intensity of an intrinsically fluorescent protein, LSS-mOrange was monitored upon irradiation with a free electron laser at two of its IR absorption peaks (11.36/9.56 microns) and at a wavelength (9.06 microns) where the protein has no absorption. The irradiation at the absorption peaks caused a reversible fluorescence intensity increase on the sec-min timescale (see figure). The fluorescence increase is not the consequence of the thermal effect of the IR irradiation because the steady-state fluorescence decreases with increasing temperature. The rates of the increase and relaxation showed Arrhenius-like temperature dependences. The reaction is pH dependent. Both 15N and deuterium isotope exchange in the protein increased the rates of the transition significantly. Importantly, both the amplitude and the rate of the observed fluorescence increase were specific to the IR irradiation wavelengths at the absorption peaks, though no significant increase was detected at the non-specific wavelength. Our results indicate that selective perturbation of structural regions in a protein with absorption specific far-infrared irradiation is possible.



2327-Pos Board B19

Collective Dynamics and Coherent Neutron Scattering in GFP

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In this work we present new data and analysis of the collective motions of fully deuterated green fluorescent protein, GFP, on the sub-picosecond timescale using coherent neutron scattering[1]. In this timescale proteins exhibit low-frequency vibrations, the so-called boson peak. The presence of a boson peak brings to mind the analogy often drawn between proteins and glassy materials where the boson peak is associated with strong "in-phase" motion of neighbor structural units. Our analysis however, revealed unexpectedly low coherence in the atomic motions of GFP; indicating a low amount of in-phase collective motion of the secondary structural units contributing to the boson peak vibrations and fast conformational fluctuations on the picosecond time scale. The latter are distinct from localized relaxational modes studied previously[2]. This finding is important as low-frequency vibrations in proteins may be connected to chemical barrier crossing events in enzyme activity. Indeed, the lifetimes of some non-equilibrium transition states are on the order of tens of femtoseconds, a relevant time scale for the deformations associated with these low-frequency vibrations. Additionally, boson peak motions have been connected to the rigidity of secondary structural units in proteins[3] and it will be interesting to see in the future how coherence differs in various classes of proteins.

1. J.D. Nickels et al. *Biophysical Journal* 2013 (Just Accepted).
2. J.D. Nickels et al. *Biophysical Journal* 2012, 103 (7), 1566-1575.
3. S. Perticaroli et al. *Soft Matter* 2013, 9, 9548-9556.

2328-Pos Board B20

Time-Series Analysis of Molecular Dynamics: Conformational Change and Dynamics of Collective Behavior

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Proteins have complex energy landscapes because of their specific three-dimensional structures determined by the heterogeneous interactions between