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 phenomena. 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-Dimensional 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 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

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 submicrosecond time resolution. Two-dimensional spectroscopy is a new optical technique that measures protein structural 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 z/b 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 beta-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 ACPB Determined by NMR Chemical Shifts and Metadynamics

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NMR Chemical shifts have recently gained renewed attention as a probe for both the structure and the dynamics of proteins. The structure and the dynamics of