**3888-Pos**

**Effect of Trifluoroethanol on the Tryptophan Side Chain Orientation in the Hydrophobic Core of Troponin C Studied by NMR and Time Resolved Fluorescence**

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Several NMR studies have shown that co-solvent trifluoroethanol (TFE) does not perturb the overall three-dimensional structure of proteins. We have inserted a single tryptophan (F77W) in the hydrophobic core of the N-domain of cardiac troponin C (cNTnC), and determined the structure of the mutant with and without TFE (Julien et al., Protein Sci. 2009 18:1165-74). Interestingly, the position of the tryptophan side chain orientation was shown to be in opposite directions. We have monitored the effect of TFE on the tryptophan rotamer population using 13C-HSQC spectra and used a full lineshape analysis to quantify the rate of the conformational exchange.

To further characterize this phenomenon, we have used Time Correlated Single Photon Counting experiments as a function of the TFE concentration. The time dependence could be fitted very well with three lifetimes in the wavelength region from 320 to 380 nm, and global analysis was further used. Addition of TFE (up to 19%) causes a gradual decrease of the lifetimes, due to dynamic quenching with very low quenching constants between kq = 0.1 to 0.01 M-1 ns-1. The amplitude fractions of the lifetimes change upon addition of TFE. At 340 nm, the amplitude fraction of the long lifetime (5.9 ns) increased from 13 to 29%, while that of the middle lifetime (3.3 ns) decreased from 63 to 50%. The short lifetime changed only to a limited extend. These data indicate that the change in the tryptophan indole position (different rotameric state) upon addition of TFE, as observed in NMR, is reflected mainly in the amplitude fractions of the different lifetimes. This is consistent, in this case, with the interpretation of lifetimes linked to rotameric states of tryptophan.

**3889-Pos**

**Single Molecule Studies of Polymerase η DNA Interaction with TIRF Microscopy**

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All cellular organisms depend on DNA molecules for the long-term storage of their genetic information. One rather obvious prerequisite for preserving such large amounts of information is a mechanism able to make fast and error proof duplicates despite the presence of damages. The importance of DNA damage repair for a cell's healthy growth becomes evident when considering that there are between 1000 and 1000000 induced DNA lesions in a cell per day.

Failure to correct those lesions can lead to serious medical conditions such as Xeroderma Pigmentosum. There for a special class of Polymerases capable of transcribing through such a lesion evolved. One such polymerase is Polymerase η. However, in the case of cancer treatment where such lesions are intentionally induced by chemotherapeutic agents to suppress cancer growth it would be desirable to block the repair mechanism of the low fidelity error prone Polymerase η.

There for, to gain insight into the dynamics of the underlying process of lesion-replication, we performed single molecule Förster Resonance Energy Transfer (FRET) experiments with an Total Internal Reflection Fluorescence Microscope (TIRFM). In the assay used, we avoid the difficulties of protein labelling by measuring the induced conformational changes of the template DNA by protein binding. Through these experiments, we where able to obtain information about single molecule binding kinetics of Polymerase η in the presence or absence of nucleotides and also about processivity on different DNA templates. The findings will be contrasted with other high fidelity polymerases such as the Klenow fragment.

**3890-Pos**

**Investigations of Calmodulin Conformations Resolved by Single Molecule Microscopy**

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Measurements of the distance between two dye molecules covalently linked to the calcium signaling protein calmodulin (CaM) have been previously performed by our group to investigate the conformations of CaM in solution. It was shown that calmodulin exists in a wide range of distinct conformations whose amplitudes depend upon free calcium concentrations (1). Currently, we are investigating affects that the choice of dye pair or labeling site has on measured conformations. This is done using an alternating laser excitation (ALEX) single molecule microscope system that has been custom built in our laboratory. Time correlated single photon counting in bulk samples is used to determine the time resolved anisotropy of the dye pair and the orientational mobility of each dye. Analysis of burst measurements using interphoton time burst selection criteria and the probability distribution analysis reveal a wide range of CaM conformations. Conformational analysis is performed using both discrete states and the maximum entropy method. The maximum entropy method reveals the most probable underlying conformational distribution that fits our data. Finally, we are investigating fluorescence fluctuations within CaM conformations using conformationally sorted fluorescence correlation spectroscopy.

1. Slaughter et al., J. Phys. Chem. B, 2004, 108, 10388-10397

**3891-Pos**

**A FRET-FLIM Study Reveals the Interaction between ALCAM and Actin as a Potential Regulator of ALCAM Binding Activity**

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Interactions between T cells and antigen-presenting cells represent the first step in the induction of an adaptive immune response. CD6 is a cell surface receptor expressed on mature T cells that specifically binds to activated leukocyte cell adhesion molecule (ALCAM). It has been shown that CD6 and its ligand ALCAM are actively recruited to the antigen-induced DC-T cell contact zone and that CD6-ALCAM interactions are also required during the proliferative phase of the T cell response. The molecular mechanism controlling ALCAM mediated interactions still remains unclear. Specifically, how the cytoskeleton dynamically regulates ALCAM binding activity at the cell-cell contact remains unknown. Transient cotransfection with Actin-RFP of a K562 cell line stably transfected with ALCAM-GFP was performed in order to investigate by FRET-FLIM the interaction between ALCAM and Actin. By measuring the donor fluorescence lifetime (GFP) in the absence and the presence of acceptor (RFP) the FRET efficiency and the distance between donor- and acceptor- labeled proteins were estimated. This FRET-FLIM study demonstrates the interaction between ALCAM and Actin (Figure 1) and opens the door for further investigation of the role of ALCAM-Actin interactions in the formation and stabilization of the immunological synapse.

**3892-Pos**

**Surface Diffusion of Cellulases on Cellulose Fibrils Studied through Fluorescence Spectroscopy**

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Cellulases are enzymes that can depolymerize cellulose, producing soluble sugars. Depending on their activity, cellulases can be classified into endocellulases, which randomly break intermediate glucosidic bonds, or exocellulases, which cleave bonds at the ends of cellulose chains. In addition, cellulases can be processive and cleave multiple bonds in succession. The current view of the mechanism for cellulase binding, diffusion along cellulose, and catalytic activity has been mostly derived from experiments performed in bulk. This has led to the generalized assumption that cellulases have multiple binding states: weak binding where the enzyme surveys the cellulose surface without catalysis, strong binding with or without catalysis, and processive displacement. Because the topography of lignocellulosic substrates is highly heterogeneous, the types of binding are further convolved by the availability of binding sites and reduced accessibility due to pore structure. Thus, experiments that elucidate surface diffusion behavior can significantly contribute to understanding how cellulases depolymerize cellulose and to improve the efficiency of saccharification processes by targeting limiting steps in binding, diffusion, and catalysis.

To study the characteristic interactions between cellulases and cellulose, we have employed fluorescently-labeled cellulases in conjunction with labeled or unlabeled cellulose and various fluorescence microscopy methods. In particular we have studied long-range surface diffusion characteristics for Thermobifida fusca cellulases Cel5A, Cel6B, and Cel9A through confocal FRAP and short-range displacement through single molecule tracking. In order to simplify the interpretation of results, we have used bacterial microcrystalline cellulose fibrils immobilized on glass surfaces through ''molecular combing'' and incubated with the different cellulases. The experiments presented have

been conducted at a range of temperatures that affect the binding and catalytic activities. Our findings challenge traditional assumptions about the role of long-range surface diffusion and offer a glimpse on the processivity of cellulases.

**3893-Pos**
**Investigation of the Effects of Cellulose Morphology on Synergism in Cellulase Mixtures using Quantitative Fluorescence Microscopy**
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Efficient and economic conversion of plant cell-wall materials into fermentable sugars is the principal technical challenge in making biofuels an important part of our energy future. At the most fundamental scale, cellulose hydrolysis occurs when cellulases bind to cellulose polymers and hydrolyze the β-1,4 linkages between glucose monomers. However, gaining access to polymers requires that cellulases diffuse and bind to complex three dimensional structures such as cellulose microfibers, mats, and particles. In addition, cellulases exhibit different binding characteristics depending on their reactive domains. Though individual cellulases hydrolyze cellulose relatively slowly, mixtures of cellulases and other plant cell wall degrading enzymes act synergistically to enhance rates and extents of hydrolysis. Elucidating the molecular mechanisms that give rise to synergistic behavior is an important research goal. A major question is to what degree does cellulose morphological structure - particle shape, size and pore structure - influence accessibility to cellulose polymers and how this accessibility changes with different cellulases.
To investigate the effects of cellulose macrostructure on synergism, pure fluorescently labeled populations of cellulases Cel5A, an endocellulase, and Cel6B, an exocellulase, were applied to cellulose immobilized in the form of mats, bundles and isolated cellulose fibrils. Cellulases were labeled with one of two different fluorophores and purified for a known degree of labeling. Cellulase binding on cellulose was recorded using time-lapsed fluorescence microscopy with images taken at defined time intervals. Local cellulase concentration on the cellulose fibrils was estimated using a standard curve obtained from known fluorophore concentrations. Binding kinetics curves were established for mixtures of these two cellulases to observe the time scale of cellulase diffusion and to observe how diffusion determines what fraction of each cellulase can occupy the system space to react synergistically.

**3894-Pos**
**Live Cell Fluorescence Analysis and Mathematical Modeling of Dual Mechanisms of Cdc42 Recycling in Yeast: Relationship of Internalization Rate and Morphology**
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Maintenance of robust cell polarization is a pre-requisite for oriented growth or motility biological systems. Cdc42 is a Rho-GTPase that, once polarized, organizes the deposition of growth materials to the polarized site. Previous studies in yeast have found that after initial polarity establishment, the individual molecules of Cdc42 are rapidly exchanged between the membrane and the cytosol. Despite these rapid dynamics, the site of Cdc42 accumulation is somehow held rigid to allow for polarized growth. We show that after initial symmetry breaking, Cdc42 is dynamically maintained at the polar cap through two recycling mechanisms: actin-mediated transport / endocytosis and cytosolic recycling through the Rho-GDP dissociation inhibitor (GDI) Rdi1. However, the mechanism by which the spatial relationship of these dual recycling pathways is controlled is unclear. We combined detailed live cell fluorescence measurements, including FRAP, iFRAP and fluorescence cross-correlation spectroscopy, with a thorough mathematical model to examine how dual recycling pathways of Cdc42 in yeast work together to shape the Cdc42 membrane distribution. We find that in order to recapitulate the steady-state membrane distribution of WT Cdc42, the dual recycling pathways must employ overlapping delivery windows of similar size. Interestingly, the modeling of live cell fluorescence data reveals that Rdi1-mediated tuning of a single dynamic parameter, internalization rate inside the delivery window, is sufficient to explain differences in the Cdc42 cap distribution between yeast cells destined for different morphogenic fates. These changes in Cdc42 cap distribution are correlated to the observed morphogenic differences under these conditions.

**3895-Pos**
**Self-Referencing Differential Spectroscopy Analysis in Breast Translational Research**
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A self-referencing differential spectroscopy analysis approach has been developed for broadband near-infrared (NIR 650-1000 nm) absorption spectra to reveal intrinsic optical breast cancer biomarkers. Through the application of this method that accounts for inter-patient variability using the normal tissue as an internal control, we have characterized the metabolic differences between malignant and normal tissues that result from subtle alterations in molecular disposition.
From a pilot study of 15 cancer patients performed in 2007, absorption signatures, not arising from the individual abundance in the four major chromophores (lipid, oxy-hemoglobin, deoxy-hemoglobin and water), have been demonstrated to successfully differentiate the normal and malignant tissues. Based on the data acquired from a NIR Diffuse Optical Spectroscopy Imaging instrument, specific spectral signatures containing specific NIR absorption bands are located in regions at about 760, 930, and 980 nm indicative of lipid biomarkers or water in abnormal state. The shape of the fingerprint spectra, namely specific tumor component (STC) spectra, is highly reproducible and exhibits consistent and particular wavelength-dependent characteristics. STC index algorithm was set up to quantitatively computing the residual due to components that are unaccounted for by the basis spectra.
A 61 subject retrospective study aiming to distinguish between benign and malignant breast tumors was carried out in 2008 on top of previous findings. By converting the observed molecular dispositions into a simple index (malignancy index) derived from a weighted wavelength analysis to maximize the differences between the benign and malignant tumors, two types of tumors were stratified with 95% sensitivity, 89% specificity, 91% positive predictive value, and 94% negative predictive value. The observation of pathology specific spectral signatures provides a potentially substantial method for differential diagnosis and monitoring response to neoadjuvant chemotherapy or hormonal therapy.
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**3896-Pos**
**Determination of Regional Diffusion Coefficients of Fluorescent ATP in Rat Cardiomyocytes**
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Results from several experiments indicate the existence of restrictions to diffusion of ADP/ATP in permeabilized rat cardiomyocytes. Decreased rate of diffusion is necessary to explain measurements of kinetics of respiration, sarcoplasmic reticulum loading with calcium, and kinetics of ATP-sensitive potassium channels. Determining intracellular diffusion coefficients allows for estimation of the effect of these restrictions on bioenergetics. Previously we extended raster image correlation spectroscopy (RICS) methods and demonstrated the existence of anisotropy in the diffusion of fluorescently labeled ATP in rat cardiomyocytes. Specifically, longitudinal and transverse diffusion coefficient was, respectively, ~2 and ~3.5 times smaller than in solution. We attempted to extend our method in order to construct regional maps of diffusion coefficients. Our efforts using a commercial confocal microscope failed due to shortcomings in protocol automation, photon detection and noise properties at high scanning speeds. In order to solve these issues, we custom built a confocal microscope and wrote software to automate the protocol for performing RICS measurements with varying scan speeds and rotation angles. During calibration, we demonstrated that the direction of the scan can influence autocorrelation function calculated according to RICS protocols through non-symmetric point spread function. With the new system, we have been able to measure regional diffusion coefficients in permeabilized rat cardiomyocytes. As expected, there is a sharp change of the diffusion coefficient on the border of the permeabilized cell. Interestingly, the values of diffusion coefficients estimated for the regions surrounding the cell are similar to the ones determined in plain solution. While there has been some variation in diffusion coefficients within the cell, the present precision of the method does not allow us to distinguish the diffusion coefficients in regions smaller than few micrometers. For that, further development and experiments are needed.