into the inner workings of cells. Often, high resolution structures of individual subunits are available, which can be used to assemble an atomic-level model of the complete system. To this end, various docking strategies have been developed. Regardless of the underlying docking algorithms, a major distinction can be made between interactive and fully automated docking.

Interactive docking allows a scientist to visually dock a structure into the experimental data. This task can be aided by software hints in the form force-feedback or other methods of guidance. However, without knowledge of global fitness scores, the results still remain subjective. Fully automated exhaustive search methods, on the other hand, perform global searches for the most likely docking positions. This compute-intensive approach is performed offline, with little input by the scientist. A major hurdle in this method is the difficulty of automatically identifying correct docking locations due to the low contrast of the available scoring functions. Additionally, important steric effects are difficult to incorporate into automated schemes.

Our new interactive docking approach aims to combine the best features of both methods outlined above. First, an (offline) exhaustive search is performed. The user then interactively isolates candidate docking locations, taking both their global docking score and any steric effects into account. Finally, candidate solutions are automatically refined.

The present docking methodology allows the user access to significantly more information during interactive docking while avoiding problems associated with fully automated approaches. Given the recent trend to study larger and larger macromolecular complexes, this new methodology provides a crucial tool for structural biology.

1583-Pos Automated Collection and Processing of Image Tilt Pairs for Single Particle Cryo-electron Microscopy

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Board B559

One of the remaining challenges for solving a new macromolecular structure by single particle cryo-electron microscopy (cryoEM) is obtaining an initial 3D model. The only robust approach for obtaining an initial model is to acquire two images in a microscope where each image is tilted differently relative to the camera. Then, from the image tilt pairs, particles are selected and processed either by the random conical (RCT) or orthogonal tilt reconstruction (OTR) methods to reconstruct an initial 3D volume. Currently, the method for obtaining tilted image pairs of single particles is a tedious and time-consuming process involving three major steps:

1. collecting tilted image pairs that provide significant overlap,
2. picking the particles from the electron micrographs, and
3. relating the picked particles from one image to its other tilt pair.

Classically, particles are picked manually side-by-side while making sure to correlate the individual particles in both views. An easily managed and tightly controlled pipeline is presented to streamline the manual steps in the initial model creation process. This pipeline allows users to automatically collect tilted image pairs and integrates modern particle picking methods to semi-automatically pick particles from both images simultaneously. Our overall goal is to provide a set of tools and procedures that will facilitate and automate the process for any new or unknown macromolecular structures.

This work was supported by National Resource for Automated Molecular Microscopy and NIH grants RR17373 and RR23093

Fluorescence Spectroscopy - I

1584-Pos Peak Amplitude Analysis As A Simplified Version Of The Method Of Photon Counting Histogram For Estimation Of Binding Of A Probe To Artificial And Natural Nano-particles

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Board B560

Photon counting histogram (PCH) method represents a powerful version of fluorescence correlation spectroscopy (FCS) applied in the case of a mixture of molecules having similar diffusion coefficients. The drawback of this approach is a complicated mathematical analysis of the data. In the present work we derived a simple analytical equation describing the statistics of the brightness of identical fluorescent particles reflecting their random walk in the confocal volume. The experimental data were obtained under stirring conditions which increased the number of events by about three orders of magnitude thus substantially enhancing the resolution of the method. The approach was tested with fluorescent spheres of different sizes and also binding of rhodamine to latex particles. The data suggested Gaussian-Lorentzian nature of the observation volume. The method allowed us to analyze the brightness of a mixture of particles of two types. The binding of tetramethylrhodamine ethyl ester (TMRE) to isolated mitochondria either in energized or deenergized states was determined by the new procedure and compared with the results obtained by traditional methods. The approach seems to be able to describe the heterogeneous mixture of isolated mitochondria under different conditions.

1585-Pos Numerical Simulations Of The FCS Decays Of A Donor-Acceptor Labeled Macromolecule: Taking Into Account Changes In The Diffusion Constant Of The System

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Board B561

In previous work we have developed a new technique for extracting kinetic rate constants from the auto and cross correlation decays in FCS-FRET experiments. We considered the case of a donor-acceptor labeled molecule which undergoes a conformational change between two conformations. We showed how the ratio between the auto-correlation of the donor (or acceptor) to the cross-correlation of the FRET pair eliminates the diffusional part of the auto and cross-correlation, isolating the kinetic terms of interest. This method thus eliminates the need for a separate experiment to characterize diffusion, and hence also eliminates artifacts due to the unavoidable differences in experimental conditions between two independent experiments.

Unfortunately, this method fails if the diffusion constants of the two conformations are significantly different, since the correlation functions cannot be derived analytically in this case. In this instance, curve fitting and determination of parameters becomes impossible without introducing significant errors. To address this issue, we have used numerical methods to generate simulated auto and cross correlations in the case of non-equal diffusion constants. Data were compiled for various values of the FRET efficiencies, reaction rates, and diffusion constants, yielding many possible scenarios. Comparison of these data to the theoretical correlations in the case of equal diffusion constants permits identification of conditions where use of these theoretical expressions ceases to be justified.

1586-Pos Protein Mobility in Crowded Dextran Mixtures

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Board B562

Protein mobility *in vivo* deviates from simple Fickian diffusion suggesting multiple diffusive mechanisms. One can envision that these deviations result from protein-environment specific interactions and non-specific interactions such as macromolecular crowding from mobile and immobile obstacles. These interactions depend, in part, on the relative size of the protein to that of molecules it encounters. Using Multi-Photon Fluorescent Correlation Spectroscopy (MP-FCS), this study addresses how the crowding level and relative sizes of the protein to mobile obstacles affect diffusion using a multiple component model and the maximum entropy method fitting (MEM-FCS). The protein under study is green fluorescent protein labeled calmodulin (eGFP-CaM), whose mobility is of great interest in many cells including in neuronal dendritic spines for understanding synaptic plasticity. Mixtures of mobile obstacles up to 30% (w/v) of the glucose polymer dextran sizes 0.25 to 12 times the size of eGFP-CaM (43 kDa) were used to simulate crowding. Dextran behavior follows polymer phenomenology but is stiffer since its viscosity shows no dependence on shear rate which has been attributed to its branching. Nanostructuring is present more often for mixtures composed primarily of larger dextrans, but this accounts only for $<1\%$ of a probe's mobility. When the nanostructuring is not present or can be ignored, we find that probe diffusion in dextran mixtures follows an exponential slowing with increased crowding which can be described by a single parameter β that depends on its component dextran size composition. Our results indicate that single component diffusion is more favored in concentrated mixtures which include viscogen sizes smaller than the probe. This work indicates that multiple diffusive components could be created or suppressed solely through size and crowding considerations.

1587-Pos Brightness Analysis with Fluorescence Fluctuation Spectroscopy in the Presence of Hydrodynamic Flow

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Board B563

Fluorescence fluctuation spectroscopy (FFS) provides information about transport parameters, concentration, and interactions of fluorescently labeled molecules. Another important FFS parameter is brightness which provides information about the composition of a protein complex. Here we consider the application of FFS to large complexes, such as viruses and vesicles. A drawback of FFS applications to large particles is the long data acquisition times required to achieve good statistics due to the low concentration and slow diffusion typically encountered in these systems. Translating the sample relative to the beam ensures that more particles pass through the observation volume, which overcomes the challenge of long data acquisition times. We implement this approach by flowing particles through microfluidic channels and investigate the influence of flow speed and particle size on FFS parameters. In addition, we apply the technique to study viral-like particles (VLP) that contain fluorescently labeled Gag proteins. The influence of flow on the brightness of VLPs is examined in order to determine the Gag copy number of VLPs in the presence of flow.

This work is supported by National Science Foundation (PHY-0346782) and a grant-in-aid (20885) from the University of Minnesota.

1588-Pos N&B Fluctuation Analysis Of Paxillin Reveals Mechanisms Of Adhesion Assembly And Disassembly

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Board B564

The number of particles (N) and brightness (B) analysis is used to measure the aggregation state of a paxillin-EGFP construct in CHO-K1 cells. This analysis is based on the intensity fluctuations measured at each pixel of an image sequence and has been developed for both photon counting and analog detectors. While the N&B analysis is complementary to the RICS (Raster-scan Image Correlation Spectroscopy) analysis developed few years ago, it provides pixel

resolution information about the number and size of protein aggregates from relatively few images of live cells independently of the diffusion of the molecule. Our analysis shows that paxillin exists as a monomer in the cytosol and forms relatively large aggregates, the size of which can depend on the particular adhesion. In large adhesions, paxillin is relatively immobile and exchanges slowly with the surrounding population. The N&B analysis of growing adhesions show that monomers of paxillin add to the adhesion. The B (brightness) map obtained during this phase is relatively constant except in the immobile regions of the adhesion, where B tends to the limiting value of 1. When adhesions disassemble, large fluctuations are detected. The N&B analysis shows that the fluctuations at the disassembling edge of the adhesions correspond to paxillin clusters of about 5 to 12 monomers. The observation of these clusters is transient and it is predominant during the initial phase of disassembly.

Work supported in part by the Cell Migration Consortium PHSSUBUVGC 10641 (MD, AFH, EG) and NIH-P41 P41-RRO3155 (EG).

1589-Pos Spatially Resolved Camera-based TIR-FCM of Single eGFP Molecules

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Board B565

Camera-based total internal reflection fluorescence correlation microscopy (TIR-FCM) has recently been described for performing spatially resolved correlation measurements near surfaces. Here we analyzed the suitability of this method for measuring spatiotemporal dynamics of single GFP molecules using an electron-multiplying charge-coupled device camera. We found that even at sampling rates of 1200 fps with a corresponding pixel region of 16×16 Pixels or 800 fps for 32×32 Pixels it is possible to visualize and correlate the fluorescence signals of single eGFP molecules in solution. We show that even minor differences in diffusion caused by small viscosity changes of the solution can be resolved by TIR-FCM. Likewise the measured autocorrelation functions changed in a predictable manner when altering concentration of fluorescent molecules, camera binning, and the depth of the evanescent field. Thus TIR-FCM appears to be a valuable and reliable method for spatially resolved live-cell imaging of diffusion and reaction dynamics of GFP-tagged molecules at the cytosol-plasma membrane interface.

1590-Pos Probing Protein Interactions in the Cytoplasm of Cells by Fluorescence Fluctuation Spectroscopy

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Board B566

Protein interactions are a crucial component of all cellular processes. Fluorescence fluctuation spectroscopy (FFS) provides a noninvasive tool for quantifying transport processes, concentration, and protein interactions in living cells. We have previously demonstrated that brightness analysis is capable of determining both homo- and hetero-complex formation of proteins in cells. However, FFS theory implicitly assumes that the optical observation volume is not influenced by the shape of the cell. While we found that this assumption is reasonable for FFS measurements in the cell nucleus, it is problematic for experiments conducted in the cytoplasm, where we found substantially biased brightness values. To address this problem, we performed scans along the axial direction of the beam and developed an FFS model that takes the finite height of the cell into account. We used cells transfected with GFP as a model system for evaluating the capability of this method. In addition, we applied this method to determine the oligomeric state of cytoplasmic proteins, such as endophilin.

This work is supported by the National Institutes of Health (GM64589).

1591-Pos Brightness of Oligomers and Brightness States in the Presence of Photodepletion

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Board B567

Brightness analysis with fluorescence fluctuation spectroscopy is a useful tool for determining the oligomeric state of proteins inside living cells. The analysis is based on the principle that the brightness of an oligomer is proportional to the number of fluorophores in the protein complex. However, the cumulative effect of photobleaching in the small volume of cells leads to a decrease of the fluorophore concentration, which we term photodepletion. The bias introduced by photodepletion scales linearly with initial fluorophore concentration. In addition, photobleaching introduces new brightness species, because each fluorophore of an oligomer bleaches independently. Both effects bias the brightness and may lead to a misinterpretation of the fluctuation data. We introduce a model that takes photodepletion into account and verify the model by performing experiments with monomeric and dimeric protein complexes. In addition, we examine the brightness states of GFP and demonstrate the presence of a photoconversion step before bleaching. These studies are conducted with one-photon and two-photon excitation, as well as wide-field illumination.

This work is supported by the National Science Foundation (PHY-0346782) and NIH grant R01GM064589.

1592-Pos Using tunable Cy5 blinking kinetics for detection of single-nucleotide differences

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Board B568

The intriguing blinking properties of carbocyanine dye Cy5 conjugated to DNA have attracted great attention. Lately it has been reported that the long-time-scale blinking kinetics of Cy5 (from milliseconds to seconds) can be altered by the proximity of the donor dye and the presence of blue laser illumination [Bates *et al* Physical Review Letters 2005]. When using fluorescence correlation spectroscopy (FCS) to study the microsecond-time-scale photophysical properties of Cy5 labeled on DNA, we notice that Cy5 possesses intriguing fast-blinking kinetics that can be "tuned" by altering the numbers of unpaired guanines in Cy5's close proximity. The observed fast-blinking kinetics are in fact resulted from the isomerization and back-isomerization of Cy5. It has been suggested that Cy5 can stick to the DNA and it is the rate of unsticking that determines the observed isomerization rate [White *et al* J. Am. Chem. Soc. 2006]. Given these considerations, we hypothesize that Cy5 in *trans* conformation (*trans* is the normal, fluorescent state while *cis* is a dark state) couples better to nearby, unpaired guanosines. Not only does this coupling slow down the isomerization of Cy5, but it also slightly enhances the Cy5's fluorescence emission since the dye now stays in its *trans* form longer than typical. We further demonstrate that this newly discovered "tunable" blinking property of Cy5 can be used to differentiate DNA targets (sequences selected from the *Kras* gene) with single-nucleotide differences. This discrimination relies on the design of a probe-target-probe DNA three-way-junction (3WJ) whose base-pairing configuration can be altered due to a single-nucleotide substitution on the target. Reconfiguration of the three-way-junction alters the Cy5-guanosine interactions, therefore resulting in a measurable change in Cy5's mean relaxation time. This pioneering work opens the doorway of using tunable fast-blinking properties of Cy5 in bioanalysis.

1593-Pos Analysis of Solution Phase Antibody-Antigen Interactions Using Time Integrated Fluorescence Cumulant Analysis

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Board B569

We explore the use of molecular brightness analysis in detection and characterization of antibody binding. To determine brightness, we use time-integrated fluorescence cumulant analysis (TIFCA). TIFCA, a recently developed fluorescence fluctuation spectroscopy technique, is sensitive not only to molecular brightness, but also to diffusion rates of molecules passing through the observation volume. We apply TIFCA to study binding of monoclonal antibody 106.3 to brain natriuretic peptide (BNP) N-terminus labeled using Alexa488. Binding of BNP to mAb 106.3 results in a substantial change in the diffusion coefficient due to the size of the complex. Also, because the antibody has two binding sites, the bound molecules have a normalized brightness of either one or two. These changes in both diffusion and brightness make the system ideal for study using TIFCA. We show results from titration experiments performed to resolve the number of single and double bound molecules within the system. A comparision will be made between TIFCA and FCS to determine the affinity of mAb 106.3 to BNP.

1594-Pos Analyzing TBP binding to DNA using HMM Analysis

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Board B570

Binding of TBP to the TATA sequence of a eukaryotic gene is the initiating step in DNA transcription - the first process in protein expression. Binding of TBP into the minor groove of the double helix takes place in several discrete steps and yields a complex bending of dsDNA. To analyze the dynamics of the TBP binding process, we developed an in vitro single molecule assay based on a single pair Fluorescence Resonance Energy Transfer (FRET). A single cysteine mutant of TBP was labeled with a FRET-donor and a 70 bp dsDNA, which contains a TATA sequence in the middle of the strand, was labeled near the TATA box with a FRET-acceptor. Monitoring the specific TBP-TATA binding revealed a dynamic process with discrete FRET states which last several seconds, indicating the existence of different intermediate conformations. In order to evaluate the kinetics between them, we utilize a Hidden Markov Model (HMM) approach. HMM is a unbiased statistical method that can be used to extract the FRET values, transition rates and number of states based on machine learning.

We have developed a HMM algorithm and applied it to our TBP binding data.

1595-Pos Cell Migration In Collagen Matrices: Assessment Of Local Stiffness By Fluctuation Spectroscopy

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Board B571

We studied the migration of individual tumor cells, derived from the human glioma cell line ACBT that were implanted into a 3D gel matrix of collagen type I. Depending on the density of the matrix, cells can migrate in this 3D tissue model. The properties of the matrix are crucial for the migration of the cells. The cells and the collagen fiber network where imaged using a confocal microscope in the reflection mode so that the sample could be observed for a long period of time without bleaching. We developed a method based on image correlation spectroscopy and on local measurement of thermal fluctuations to determine the average size and the local stiffness of the collagen fibers. Intensity fluctuations were generated by the flickering of the reflections from the fibers. The reflected intensity from a single point in the fiber fluctuates due to fiber position fluctuations. We found a correlation between the fiber diameter and the fluctuation spectrum. Using continuous acquisition for several hours we determined the displacement of the fibers close to the cell as the cell forces its way in the 3D network during the migration process. We also observed changes in the fluctuation spectrum at specific locations probably due to the formation of cellular adhesions.

This research was supported by the National Institutes of Health (PHS-9RO1-EB00559 and NTROI-1U54CA105480)

1596-Pos A New Approach to Global Analysis of Fluorescence Fluctuation Spectroscopy Data

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Board B572

Fluorescence fluctuation spectroscopy is an experimental technique that relies on the analysis of fluctuations to characterize ensembles of molecules. The two most widely known methods from this family are fluorescence correlation spectroscopy (FCS) and photon counting histogram analysis (PCH) also known as fluorescence-intensity distribution analysis (FIDA). These two methods complement each other and are used to study dynamic processes (FCS) and association reactions (PCH/FIDA). Much of the current research in the field of FFS is focused on bridging the two and developing global data analysis methods that allow one to simultaneously characterize molecular states and dynamics of transitions among them. We describe a new approach to this problem that stems from the idea of higher order correlation function analysis. Briefly, the method preserves approximately logarithmic time scaling inherent to modern multi-tau correlators but complements the time axis with a brightness dimension, i.e., the number of photons per sampling time interval. Using such composite analysis, we are able to determine the number of molecules, brightness per molecule, and characteristic diffusion time in a model system. We believe that this new global analysis tool can be useful in situations where both changes in brightness and in diffusion coefficient occur as a result of a chemical reaction. Such is the case, for example, in association reactions with macromolecules.

1597-Pos Scanning Fluorescence Correlation Spectroscopy on Membranes

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Board B573

Here we demonstrate the capacity of scanning fluorescence correlation spectroscopy for accurate measurements of diffusion and binding parameters on model membranes and *in vivo*. The high flexibility, reduction of photo bleaching and a remarkable signal to noise ratio combined with an intrinsic robustness against instabilities and artifacts extend the use of FCS to demanding systems.

Scanning two focus FCS measures diffusion without the need for calibrating the detection volume and therefore greatly enhances the accuracy compared to traditional FCS. Scanning dual color cross correlation with alternating excitation permits binding studies without the risk of false positive results due to membrane movements or spectral cross talk.

With the help of scanning FCS we could measure extremely slow diffusion in yeast cell membranes. We also applied scanning two focus FCS to measure the diffusion of the mRFP tagged fibroblast growth factor receptor 1 (FGFR-1) in the membranes of living zebra fish embryos and dual color scanning FCS to study its binding affinity to the GFP-labeled ligand *in vivo*.

Applied on horizontal membranes, scanning FCS allows for fast and accurate calibration free diffusion and concentration measurements.

The simple implementation in a commercial laser scanning microscope should help to establish scanning FCS as a standard method for membrane studies.

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1598-Pos Probing the Movement of HIV Virions in Cervical Mucus

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Board B574

Mucus is a hydrogel (~95% water) that serves a multitude of functions. To reach the mucosa many pathogens, including HIV, have to penetrate through the mucus, whose role in protection

against pathogens needs to be elucidated. In this study, we compare the diffusion of inactivated, fluorescently labeled (GFP and Alexa488) HIV virions with other fluorescent probes (phycoerythrin, polystyrene beads) through samples of natural untreated cervical mucus provided by healthy donors. We apply fluorescence correlation spectroscopy (FCS) and time-resolved fluorescence confocal microscopy (FCM) to assess this motion. When mixed with the mucus the virions appear to be trapped and their local motion is wiggly and oscillatory on millisecond time scale. In contrast, phycoerythrin proteins (~10.2 nm) appear to diffuse freely, although with a three-fold slower rate compared to that in the buffer. However, the polystyrene beads with diameters between 28 and 63 nm appear to bind rapidly and very tightly to the mucus, revealing filamentous macroscopic patterns of the mucus and its spatial heterogeneity with relatively large mesh sizes (~microns). These results indicate that the movement of the probe particles (virions, beads, phycoerythrin) is not solely governed by their size, and that specific interactions between the particles and the mucus are likely to be playing a role in this dynamics. Deciphering mechanisms that determine the pattern of this movement may be important in understanding aspects of HIV transmission.

1599-Pos FCS Studies Of c-myc : Endogenous Vs. Tranfection-induced Mobility

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Board B575

c-MYC is the classic product of a proto-oncogene. It is carefully regulated and usually expressed at very low levels (few nM) in the cell. It has been demonstrated to regulate expression of many genes and controls cell proliferation. We report the measurement of c-MYC-eGFP expressed from its natural locus in the nuclei of primary mouse fibroblasts using Fluorescence Correlation Spectroscopy. FCS is aptly sensitive to the endogenous nM c-MYC present in the nucleus. The experimental data is fit to two diffusing populations, an almost immobile fraction and another large (apparently ~Mda), but much more mobile. When increasing the total concentration of c-myc-eGFP with transient transfection(s), total c-MYC levels increase steadily and the 'mobile' pool rapidly climbs toward 90% of the total c-myc-eGFP (after about 100nM), while the immobile component concentration remains near endogenous levels, thus decreasing from 50% to only a few percent of total signal. We will characterize these fractions at different expression levels under various conditions. The importance of recognizing the small, easily saturated endogenous pool of binding sites in regulation will be discussed.

This research was supported by the Intramural Research Program of the NIH, NHLBI and NCI.

1600-Pos Quantitation of Biomolecular Interactions By Single Wavelength Fluorescence Cross-Correlation Spectroscopy (SW-FCCS) *in vivo*

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Board B576

Single wavelength fluorescence cross-correlation spectroscopy (SW-FCCS) can be used to study the dimerization or binding of two molecules labeled with spectrally different fluorophore. The concentrations of the molecules that are bound or free can be calculated from SW-FCCS measurements. In theory, by plotting the product of the concentration of free molecules [C1] $\times$ [C2] against the concentration of complexes [C12], the dissociation constant (Kd) can be obtained as the slope of the expected straight line. However, in the case of either unlabeled competitors or non-fluorescent proteins, the expected curve is non-linear. This work thus aims to investigate the influence of the fluorescent properties of fluorescent proteins on the minimal and maximal expected cross-correlations and on the possibility of quantitation of biomolecular interactions. For this purpose we model different situations and compare the results to measurements taken on different fluorescent proteins (EGFP, mRFP, mCherry) and fluorescent proteins in different cellular localization (membrane versus cytosol). As a standard negative control with no expected binding we use EGFP co-expressed with either mRFP or mCherry in the cytosol of CHO cells. As a positive control we use EGFR-mRFP, EGFR-mCherry, and EGFP-Epidermal Growth factor Receptor -mRFP (EGFP-EGFR-mRFP) constructs and compare the results to theory to calculate the number of EGFP, mRFP, and mCherry molecules in fluorescent and dark states. As a test system for molecular interactions we used the protein pair EGFP-IQGAP1 co-expressed with mRFP-Cdc42. The measurements show clear differences form the negative control, proving binding. However, to determine an in vivo Kd, photochemical properties of the fluorescent proteins need to be taken into account.

1601-Pos Simultaneous Flim Measurement Of The Decay Of The Donor And Acceptor In A Fret Pair

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Board B577

We have developed a method based on the phasor approach to evaluate globally the decay of the donor and of the acceptor in a FRET pair in images of cell expressing a construct of EGFP and mRFP. Data where acquired with a spectral detector in the photon counting mode using the Becker and Hikl module. In the phasor plot,

the donor and the acceptor phasors move along specific trajectories depending on the FRET efficiency. The superposition of these two contributions results in phasors aligned in a straight segment. Therefore, the measurements at different emission wavelengths should give phasors along this segment. The experimental position of the segment in the phasor plot and its slope provide a unique solution in terms of the efficiency of transfer, amount of donor without acceptor, amount of direct excitation of the acceptor and background contribution. This solution could in principle be obtained with a global fit of all the data set. Instead, in the phasor representation the solution is obtained by simple inspection of the phasor plot of the sum of all experiments at the different emission wavelengths. The uniqueness of the solution can be established by visual inspection. The phasor approach is particularly useful for experiments done in cells in which the spatial separation of pixels with different FRET efficiencies could be resolved.

1602-Pos Fretting About FRET

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Board B578

FRET has been an important tool in structural biology for over three decades and is now widely applied to complex systems using modern techniques that could only be imaged by its progenitors. These pioneers carefully examined a number of assumptions that must be made when analyzing FRET data. Despite the fact that contemporary systems and techniques are often very different then those used thirty years ago, current FRET studies often rely on these same assumptions without validation. We have utilized computer simulation methods to examine a number of these assumptions. In particular, we present the results of molecular dynamics simulations and quantum mechanical calculations that help to demonstrate when problems may arise from, for instance, failure of the $\kappa^2 = 2/3$ approximation, the breakdown of the ideal dipole approximation, or the presence of correlation between the motions of the donor and acceptor probes. In addition, we present a method for simulating spectroscopic observables that allows direct comparison between experiment and simulation, avoiding the need for many of the usual FRET assumptions.

1603-Pos FRET with Multiple Fluorescent Protein Acceptors

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Board B579

Förster resonance energy transfer (FRET) can be used to study protein-protein interactions inside living cells. Typically proteins are tagged with cyan and yellow spectral variants of GFP (FPs). FRET theory is based on energy transfer between a single donor and a single acceptor, and assumes that transfer occurs from the lowest vibrational level of the excited state. In live cell experiments, however, multiple acceptors might be present complicating the interpretation of FRET experiments. Furthermore, unlike traditional fluorophores, the chromophore in FPs are buried in a β barrel protein structure and are thus isolated from the influence of the external environment. This isolation can potentially alter certain aspects of their photo-physics. While a theory describing FRET in the presence of multiple acceptors has been developed, it has not been tested for energy transfer between FP donors and acceptors. DNA constructs encoding FP donors and acceptors in various different configurations and stoichiometries were constructed to test if this general theory describing FRET with multiple acceptors is applicable to FPs. Preliminary results indicate that FRET efficiencies of constructs with multiple FP acceptors were greater than predicted by theory. A possible explanation for this anomalous behavior will be discussed.

1604-Pos Structural Dynamics of SERCA and Phospholamban by Fluorescence Microscopy

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We have investigated the structure of phospholamban (PLB) and its regulation of the sarcoplasmic reticulum Ca-ATPase (SERCA) using fluorescence resonance energy transfer (FRET) on fluorescent fusion proteins expressed in living cells, and using total internal reflection fluorescence (TIRF) on the labeled cytoplasmic domain of PLB. Fusion proteins were created with either the donor fluorophore, cyan fluorescent protein (CFP), or the acceptor fluorophore, yellow fluorescent protein (YFP), attached to one terminus of the protein of interest. Both N-terminal and C-terminal fusions of CFP and YFP were made to SERCA and N-terminal fusions were made to PLB. These proteins were expressed and co-expressed in either SF21 insect cells or HEK-293 cells. In fluorescence transfer recovery (FTR) experiments, FRET was calculated from the recovery of CFP fluorescence due to photobleaching of YFP. The dependence of donor fluorescence on acceptor photobleaching showed that PLB exists primarily as oligomers in cells but binds to SERCA exclusively as a monomer. Polarized TIRF of PLB, labeled in the cytoplasmic domain helix with bifunctional rhodamine (BFR), shows that this domain lies parallel to the membrane surface. The structural and functional effects of PLB phosphorylation and mutation are under investigation.

This work was supported by NIH (GM27906) and the Minnesota Supercomputing Institute.

1605-Pos Role of DNA Sequence and Stacking Interactions on the Fluorescent and Photophysical Properties of Cy3-DNA conjugates

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Board B581

The sulfoindocyanine Cy3 is one of the most commonly used fluorescent dyes used in biophysics. Recent studies in our lab have shown that the photophysical properties of Cy3 change dramatically upon covalent attachment to DNA. These changes include an enhancement of fluorescence in both single and double stranded DNA with interesting differences between ssDNA and dsDNA being observed. This suggests an interaction between Cy3 and DNA which we further investigate in this work. Here, we explore the role of DNA sequence on the photophysical and fluorescent properties of the Cy3-DNA conjugates. Surprisingly, no changes have been observed for heteronucleotide DNA constructs but significant differences have been detected for polynucleotides. Furthermore, Cy3 in the presence of dMTPs has been studied in order to clarify the role of stacking. Results show an increase in fluorescence, similar to the enhancement noted above with DNA, for Cy3 with purines but little or no increase with the pyrimidines. Time resolved fluorescence experiments provide further evidence of the interactions of Cy3 with DNA.

1606-Pos Fluorescence-based Calcium-ion Sensing At High Hydrostatic Pressures

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Board B582

With the biosphere's average pressure at 380 atm, understanding pressure effects in biological systems is of both fundamental and applied interest. Advances in determining the molecular and genetic bases for piezophysiologic effects have been made during the last decade or so. Because calcium ions are nearly ubiquitous in intracellular signaling and control, methods for calcium-ion sensing at high pressure would be useful in the *in vivo* investigation of these effects. Here we develop high-pressure techniques for *quantitative* fluorescence-based calcium sensing using Fluo-4 and related intracellular dyes. The dyes ($<2\mu M$) are characterized in EGTA/MOPS calcium-buffer solutions (10mM EGTA, 30mM MOPS, 100mM KCl, *pH* 7.2, *pCa* 4 through 9) with fluorescence emission spectra measured at pressures up to 500 atm on a custom-built spectrofluorimeter utilizing a quartz-capillary pressure chamber. *pK* under pressure is determined using a two-state calcium-bound/unbound model for fluorophore-calcium-ion dissociation. Assuming an Arrhenius relation (appropriate for this pressure range) and using the known pressure response of the EGTA/MOPS calcium buffer as a reference, the ΔV of the Fluo-4 dissociation reaction can be determined. In addition to calcium-sensing *in vivo*, quantitative results presented here have potential applications in the broader high-pressure ion-sensing field.

1607-Pos Femtosecond Fluorescence Spectra of Human gamma D-, gamma S-Crystallin Trp Mutants: Site-Dependent Ultrafast Quenching

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Board B583

The gamma-crystallins are remarkably stable globular proteins with interesting fluorescence properties (J. Chen et al., Biochemistry 2006, *45*, 11552). Recently, we have characterized Trp in proteins with femtosecond fluorescence upconversion methods sensitive to dynamics in the .2 ~ 200ps range. We found that bulk water relaxation around Trp in ~2ps leads to spectral relaxation (X. Shen et al., J. Phys. Chem. B. 2001, *105*, 6260). Others (A. Zewail et al., P. N.A.S., 2002, *99*, 10964) then found that the average spectral position of Monellin also continued moving with ~16–30ps rates; we found, however, that ultrafast ("quasistatic") quenching of some Trp conformers, not relaxation, is responsible for this term (J. Xu et al., J.A.C.S. 2006, *128*, 1214). This is also seen in "quasistatic self-quenching" of dipeptides (R. Chen, Biochemistry 1991, *30*, 5184).

We replaced 3 of 4 Trps with Phe, leading to single-Trp mutants: W68-only and W156-only of H gamma D- and W72-only and W162-only of H gamma S-crystallin. All exhibit similar DAS (decay associated spectra) on the 50-65ps time scale, and their signature ("positive DAS") indicates dominance of fast, heterogeneous quenching. The population (amplitude) of this DAS differs among mutants. Trps 68 and 156 in H gamma D- and Trp162 in H gamma S- crystallin are buried, but water can reach amide oxygen and ring HE1 atoms through narrow channels. QM-MM simulations of quenching by electron transfer predict heterogeneous decay times from 70–500 ps that are correlated with the number of H-bonds, due to their stabilization of the CT state.

This work was supported in part by DIR, NIH, NHLBI and NSF grants MCB 0416965, MCB 0719248.

1608-Pos Conformational Heterogeneity of L-Tryptophan Revealed by Spectrally Resolved Fluorescence Decays

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Board B584

Fluorescence decay kinetics of protein is usually described as linear combination of exponentials, even in single tryptophan (Trp) proteins. Rationalizing the decay parameters, we can elucidate function-structure relationships of protein by time-resolved fluorescence spectroscopy. But the origin of such multiexponentiality has been in controversy and this hampers the quantitative interpretation of fluorescence data. At present, some interpretations are proposed for multiexponential or nonexponential decay of Trp fluorescence. One of those interpretation models is rotational/conformational isomer (rotamer/conformer) model that ground state conformation heterogeneity of Trp would be responsible for each decay component. In other models, dielectric relaxation process in the excited singlet state is given priority as a potent factor for multiexponential decay kinetics of Trp fluorescence.

We recently examined time-resolved emission spectra (TRES) of L-Tryptophan (L-Trp) in glycerol and the result of time-resolved area-normalized emission spectra suggested the existence of two emitting species. By fitting TRES of L-Trp with two spectral functions, we confirmed the distinct dielectric relaxation rates and fluorescence lifetimes of two emitting species. It is confirmed by NMR studies that L-Trp has rotamer. So we concluded that those two peaks were corresponding to rotamers of L-Trp and those rotamers have inherent electrostatic interaction with glycerol and intrinsic nonradiative properties.

These results are informative to elucidate the photophysical process of Trp and to investigate the protein structure and function using fluorescence spectroscopy.

1609-Pos Mechanism of Fluorescence Quenching of Tryptophans in Human γS-Crystallin

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Board B585

Human γS-crystallin (HγS-Crys) is a stable eye lens protein that is expressed post-natally in the outer layers of the eye lens. It must remain soluble and folded throughout the human lifetime. HγS-Crys has two homologous β-sheet domains, each containing a pair of highly conserved buried tryptophans. These tryptophans display anomalous fluorescence that is quenched in the native state with respect to the denatured state. By combination of experiments together with theoretical quantum mechanical-molecular mechanical (QM-MM) calculation, we revealed the mechanism of this quenching. Trp72 and Trp162 of HγS-Crys are highly quenched, with quantum yields of 0.013 and 0.03, respectively. Trp46 and Trp136 are moderately fluorescent, with quantum yields of 0.066 and 0.25, respectively. There is energy transfer from Trp136 to Trp162 in the C-terminal domain but not in the N-terminal domain. QM-MM simulations strongly indicate that electron transfer rates to the amide backbone of Trp162 are extremely fast relative to those for Trp136. Quenching of Trp72/162 is due to the efficient electron transfer from tryptophan indole ring to amide backbone. Additional information was obtained using time-resolved fluorescence spectroscopy. In single-Trp containing proteins, the highly quenched Trp72 and Trp162 have short lifetimes of few hundred ps. The moderately fluorescent Trp46 has long lifetime (τ~1ns) and Trp136 has much longer lifetime (τ~4ns). In the presence of the energy acceptor (Trp162), the lifetime of the energy donor (Trp136) decreased from ~4ns to ~1ns. The intradomain energy transfer efficiency is 65% in the C-terminal domain and there is almost no or little energy transfer in the N-terminal domain. The quenching mechanism of HγS-Crys is similar to human γD-crystallin reported previously (Biochemistry 2006, *45* (38): 11552–63). The backbone conformation of tryptophans in γ-crytallins may have evolved to protect the tryptophans of crystallin proteins from UV-induced photodamage.

1610-Pos A Reducing and Oxidizing System for Unprecedented Stabilization of Fluorescent Dyes

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The exquisite selectivity, sensitivity and spatial resolution obtained with fluorescence spectroscopy and imaging have led to an ever increasing number of applications. With the development of detectors approaching unity quantum efficiencies and sophisticated collection optics the bottleneck of current fluorescence microscopy are the fluorophores used, which pose severe limitations due to photobleaching and blinking. Here we present a new strategy to prevent photobleaching by recovering reactive intermediates. The universal strategy is based on the removal of oxygen and quenching of triplet as well as charge separated states by electron transfer reactions. Therefore a formula that contains reducing as well as oxidizing agents, i.e. a reducing and oxidizing system (ROXS) is used. The success of the approach is demonstrated by single-molecule spectroscopy of fluorophores from different classes, i.e. cyanines, carborhodamines and oxazines and is furthermore supported by thermodynamic considerations. Besides reducing photobleaching and allowing single-molecule observations of many minutes to hours in aqueous surrounding, the brightness of the fluorophores is substantially increased because blinking is completely eliminated. Since the ROXS concept is universally valid and applicable for different fluorophore classes it will foster the spreading of optical single-molecule and high resolution microscopies.

1611-Pos Efficient Site-specific Labeling Of Proteins Via Cysteines

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Board B587

Methods for chemical modifications of proteins have been crucial for the advancement of proteomics. In particular, site-specific covalent labeling of proteins with fluorophores and other moieties has permitted the development of a multitude of assays for proteome analysis. A common approach for such a modification is solvent-accessible cysteine labeling using thiol-reactive dyes. Cysteine is very attractive for site-specific conjugation due to its relative rarity throughout the proteome and the ease of its introduction into a specific site along the protein's amino acid chain. This is achieved by site-directed mutagenesis, most often without perturbing the protein's function. Bottlenecks in this reaction, however, include the maintenance of reactive thiol groups without oxidation before the reaction, and the effective removal of unreacted molecules prior to fluorescence studies. Here, we describe an efficient, specific, and rapid procedure for cysteine labeling starting from well-reduced proteins in the solid state. The efficacy and specificity of the improved procedure are estimated using a variety of single-cysteine proteins and thiol-reactive dyes. Based on UV/VIS absorbance spectra, coupling efficiencies are typically in the range of 70~90%, and specificities are better than ~95%. The labeled proteins are evaluated using fluorescence assays, proving that the covalent modification does not alter their function. In addition to maleimide-based conjugation, this improved procedure may be used for other thiol-reactive conjugations such as haloacetyl, alkyl halide, and disulfide interchange derivatives. This facile and rapid procedure is well suited for high throughput proteome analysis.

Fluorescence Spectroscopy - II

1612-Pos The Influence of Computational Method and Basis Set on Electron Transfer Integrals that Determine Tryptophan Fluorescence Quenching in Proteins

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Board B588

The ab initio computed values of electron transfer coupling integrals for photoinduced electron transfer from the ^1L_a excited indole ring state to a local backbone amide play an important role in QM-MM predictions of fluorescence quantum yields for tryptophan residues in numerous proteins. Recently, we have presented a hybrid molecular dynamics (MD)/quantum mechanical method with several advances, including ab initio calculations of the electronic coupling matrix element as a function of conformation for each protein studied (Callis et al., J. Phys. Chem. B **2007**, *111*, 10335). In particular, using the CIS method, we establish for the first time how three basis sets, STO-3G, 3-21G, and D95 influence the computed average values of electron transfer integrals taken along a 150 ps MD trajectory. We now turn our focus to the adequacy of these relatively simple basis sets and of the CIS Hamiltonian matrix to produce realistic coupling elements. We report how the computed values of electron transfer integrals depend on computational method and a further extension of the basis sets. The Generalized Mulliken-Hush (GMH) and direct CIS methods are compared for 20 tryptophans in 17 proteins. We find that the GMH and CIS values of electron transfer integrals don't correlate very well along the MD trajectory, but their average values span the same range of magnitude. Including polarized and diffuse functions in the basis sets barely affected the average value of electron transfer integrals, although for individual conformations variations by a factor of 10–100 were found, especially with small integrals. We conclude that the simplest basis, STO-3G, may be effectively used for computing electron transfer integrals at the CIS level, providing short term fluctuations are unimportant.

1613-Pos Prediction of Fluorescence Quantum Yields for Tryptophan: Improved Classical Sampling by Free Energy Perturbation

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Board B589

Recently we presented an ab initio method for predicting fluorescence quenching of tryptophan (Trp) in proteins [Callis et al J.Phys. Chem. B **2007** *111*, 10335]. The quenching is due to electron transfer from the indole ring in the ^1L_a excited state to a nearby amide, creating a dark CT state. The rate is given by the Fermi golden rule, $4\phi^2/h(V^2\rho_{FC})$. Surprisingly, the electronic coupling elements (V) turned out to be 1-2 orders larger than used in a previous empirical procedure (Callis and Liu, 2004). For agreement with experiment, the much larger coupling requires a much smaller Franck-Condon weighted density of states (ρ_{FC}) that is much more consistent with the expected large CT-La energy gap. The new method is still effective in predicting low and high quantum yields, but agreement is scattered for intermediate cases. The average ρ_{FC} is now much more sensitive to the shape of the extreme tail of the classical probability distribution corresponding to rare conformational fluctuations that have the smallest CT-La gaps. Previously, we assumed the classical probability distributions were Gaussian. As an improvement to the method, we report here calculations based on a more realistic classical distribution far from the equilibrium point by using the free energy perturbation (FEP) method (Warshel and King 1990). The FEP-augmented distributions are not Gaussian, and they show generally improved agreement with experiment.