water insolubility of HY, one of its major draw-backs. The aggregation and binding of HY in the presence of PVP were studied and digitized fluorescence endoscopic imaging (DFEI) was used to study the effect of the pharmaceutical formulation with the *in vivo* tumor implanted chick chorioallantoic membrane (CAM) model. The combined results from FCS and DFEI studies reveal the coordination of HY-PVP binding, HY disaggregation in the presence of PVP and strengthened HY tumor uptake selectivity. PVP is thus suggested as a potential adjuvant to previously investigated N-methyl pyrrolidone (NMP) in the HY delivery system as well as a replacement for the conventionally used albumin in the HY bladder instillation fluids preparation for clinical use. The findings were then further confirmed by FLIM studies in HY solutions, followed by measurements in MGH and RT112 cells incubated with HY and PVP solutions in dark. Fluorescence lifetime of intracellular HY showed similar trend of prolongation with the presence of PVP as that was observed with FLIM measurements in solutions. The FLIM results, supported by colocalization studies by confocal imaging with fluorescently labeled PVP, strongly suggest that PVP is uptaken in cancer cells despite the large number of contrary reports.

225-Pos Board B104
Brightness Analysis of Nuclear Receptor Interactions in a Cell-Free Expression System
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Brightness analysis with fluorescent fluctuation spectroscopy examines protein interactions in real time. The analysis is based on the principle that the brightness of an oligomer is proportional to the number of fluorophores in the protein complex. We previously applied brightness analysis inside living cells to decipher both homo- and hetero-interactions between proteins. While experiments in cells probe protein interactions in the natural environment, solution studies probe interactions between isolated proteins. In some cases we observed different results in cells than expected from solution studies. In order to identify the origin of such differences, we adapt brightness analysis to solution studies by utilizing a cell-free expression system. Cell-free expression takes advantage of genetic tags for labeling proteins while providing a much more controllable environment than the living cell. We first characterize the behavior of mCherry and EGFP in a cell-free expression system to optimize it for brightness analysis. The samples are measured in microfluidic devices using two-photon excitation. Using this system, we study the oligomerization states and interaction of nuclear receptor proteins RXR and RAR by means of brightness analysis. We determine the binding affinity of these receptor proteins for comparison with measurements made in living cells and on purified protein systems. This work is supported by the National Science Foundation (PHY-0346782) and NIH grant R01GM064589.

226-Pos Board B105
Resolvability of PCH in Two Dimensional Systems
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Although the ability of PCH analysis to resolve the components of mixtures of fluorescent molecules has been carefully studied in three-dimensional systems (Muller, Chen et al. 2000), it has not been investigated in two dimensions. We explored the characteristics of the reduced $\chi 2$ surface of two dimensional binary mixtures, specifically, the principal curvature at the $\chi 2$ minimum as a function of brightness and molecular concentration. Our results are in good agreement with the previously published results.
A potential problem can arise from errors in focusing in two dimensional systems . When data acquisition time and therefore the data record is insufficient to to resolve species in a mixture, independent information about each species, e.g., measurements of brightness, can be used to extract more accurate results. Our experiments on Giant Unilamellar Vesicles (GUVs) labeled with a single lipid analog allow us to estimate brightness variations due to focusing. Combining this information and the PCH from mixtures yield reasonable estimations of the parameters of interest
Muller, J. D., Y. Chen, et al. (2000). "Resolving heterogeneity on the single molecular level with the photon-counting histogram." Biophys J 78(1): 474-86.

227-Pos Board B106
Insights Into The Microscopic Origin Of Anomalous Diffusion From Crowded Solutions
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Subdiffusive motion of tracer molecules has been observed in many crowded environments, ranging from polymer and protein solutions to intracellular fluids. Yet, a clear understanding of the microscopic origins of subdiffusive motion and the variation of the anomaly in crowded media is still missing. To address this point, we have studied the diffusion of tracer molecules in crowded solutions with varying composition using fluorescenc correlation spectroscopy (FCS). Aiming at capturing the essential processes that lead to anomalous diffusion, we observed that none of the artificial mixtures yielded the degree of subdiffusion that has been observed for intracellular fluids, i.e. artificially crowded solutions appear too simple to account for the high degree of anomaly observed in cells. Comparing time and ensemble averages of the tracers' mean square displacement furthermore indicated that ergodicity is unbroken. Hence, the monitored diffusive process cannot be described properly by a (non-stationary) continuous time random walk.

228-Pos Board B107
Bacterial Sec Protein Transport is Rate-limited by Precursor Length: A Single Turnover Study
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An in vitro real-time single-turnover assay for the *Escherichia coli* Sec transport system was developed based on fluorescence dequenching. This assay corrects for the fluorescence quenching that occurs when fluorescent precursor proteins are transported into the lumen of inverted membrane vesicles. We found that: i) the transport kinetics were well fit by single-exponential, even when the ATP concentration was rate-limiting; ii) ATP was utilized during most of the time required for transport; and iii) longer precursor proteins transported more slowly than shorter precursor proteins. Together, these conclusions argue against a model in which precursor movement through the SecYEG translocon is mechanically driven by a series of rate-limiting, discrete translocation steps that result from conformational cycling of the SecA ATPase. Instead, we propose that precursor movement results predominantly from Brownian motion and that SecA regulates pore accessibility through ATP hydrolysis.

229-Pos Board B108
Single Molecule FRET Measurements of Dye-labeled DNA
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Single molecule FRET (sm-FRET) measurements of dye-labeled DNA molecules have become a widely-used tool over the last decade to probe the structure, dynamics and function of nucleic acids. As robust as FRET is, quantitative measurements and absolute distances are seldom extracted, mostly due to difficulties in obtaining the correct value of the transfer efficiency and the orientation factor between the dyes. Moreover, additional complications might arise from undesired interactions between the dyes and the nucleotides, or even between the dyes themselves. We present a systematic study of sm-FRET performed on an extensive set of internally labeled DNA molecules with FRET pairs at various inter-dye distances, spanning the entire dynamic range of transfer efficiencies. An automated two-color scanning confocal microscope allows us to obtain long fluorescence time trajectories at high temporal resolution, from which we extract the corrected FRET efficiencies. By fitting these values to a geometrical model of the double helix we obtain the absolute distances between the dyes as well as the orientation factor. At inter-dye distances smaller than 8 nucleotides apart we observe an increased fraction of abrupt fluorescence fluctuations that we attribute to dye-dye interactions. We note that this phenomenon can distort the apparent FRET efficiency towards lower values, consistent with previous reports. We will show single molecule time traces which have allowed us to characterize and study these interactions in detail.

230-Pos Board B109
Structural Dynamics of SERCA and Phospholamban by Fluorescence and Phosphorescence
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We used fluorescence and phosphorescence to investigate the structure and dynamics of phospholamban (PLB), and its regulation of its inhibited target, sarcoplasmic reticulum calcium ATPase (SERCA). 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. Single molecular TIRF microscopy was used to measure the translational diffusional coefficient of Alexa488 labeled monomeric PLB reconstituted into a supported lipid bilayer. The diffusional coefficient of monomeric PLB is 0.7 $\mu m^2/s$, which is consistent with its molecular weight. Time-resolved phosphorescence anisotropy of erythrosin iodoacetamide (ErIA) labeled SERCA in cardiac sarcoplasmic reticulum (SR) was measured with and without phosphorylation of PLB in presence of high and low Ca concentrations. Phosphorylation of PLB decreased the final anisotropy of ErIA labeled SERCA at low Ca, indicating decreased SERCA self-association. This supports the proposal that PLB inhibits SERCA by inducing SERCA-SERCA association, which is relieved by phosphorylation.

231-Pos Board B110

Single molecule measurements of ATP-myosin V and ADP-myosin V

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We investigate the conformations of myosin V bound to ATP and to ADP via single molecule FRET measurements. The myosin V is labeled with FlAsH in the upper 50kDa domain, and the bound nucleotides are labeled with Rhodamine 101. We have carried out two types of single molecule FRET measurements on this complex: 1) we record the transit of single molecules diffusing through the focal region of a probe laser (473 nm); 2) we record the time trajectory of each molecule while it is encapsulated within an optically trapped femtoliter aqueous nanodroplet (hydrosome). In the latter measurements, an infrared (1064 nm) optical trap holds a single hydrosome within the focal region of the probe (473 nm) beam, which fluorescently excites the single molecule contained within the hydrosome. Our preliminary results to date indicate that our single molecule FRET measurements are consistent with each other and with previous ensemble measurements.

232-Pos Board B111

Fluorescence Labeling And Purification Of Cellulases For Single Molecule Spectroscopy

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The development of highly-sensitive detectors for optical microscopy has enabled the detection of individual fluorescent molecules and allowed life scientists to probe dynamic and conformational properties of enzymes. In single molecule spectroscopy (SMS) an essential requirement is the use of bright, fluorescent moieties. In this sense, organic dyes are small molecules that can confer fluorescence capabilities without compromising enzymatic activity. However, tracking of a single a molecule labeled with a single fluorescent moiety is limited by bleaching time and the number of photons emitted by molecule per second. Thus, in SMS it is desirable to have enzymes labeled with multiple fluorescent moieties while retaining native activities.

Most organic dye labeling techniques produce mixtures of populations of molecules labeled with different numbers of fluorophores. For SMS this poly-dispersity of labeled molecules can introduce significant variability. In addition, each of these labeled populations can have properties different from the native protein or enzyme, which further complicates the interpretation of results derived from SMS. To address this we have developed methods to label and purify enzymes with a variety of organic dyes from the Alexa-Fluor family. Our approach explored labeling in free solution and solid phase. Purification methods developed to remove unbound dye were optimized for each of these labeling methods. Separation of populations of labeled molecules was performed via FPLC and optimized for each one of the enzymes labeled. Through these methods we have produced highly purified populations of cellulases Cel5A, Cel6B, and Cel9A labeled with known numbers of dyes. These populations have been characterized for their degree of labeling, location of the fluorescent moiety, and catalytic activity as compared with the native enzymes. We demonstrate the advantage of the use of fluorescently tagged cellulases with well-known physico-chemical properties through SMS measurements.

233-Pos Board B112

Metal-Enhanced Fluorescence (MEF)

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In recent years our laboratories have described the favorable interactions and outcomes of both plasmon supporting particles (Ag, Au, Cu, Zn, Ni, Cr) and substrates with electronically excited states. These favorable effects have included enhanced fluorescence emission from singlet states, S_1 and S_2, as well as enhanced phosphorescence yields from triplet, T_1, states (MEP). In addition, we have observed and described plasmon enhanced chemiluminescence intensities (MEC), as well as highly directional emission. As a result of enhanced triplet yields, we have also observed both enhanced singlet oxygen and superoxide anion yields.

These favorable influences on the photophysical properties of close proximity excited states to plasmon supporting substrates/particles has led to wealth of biochemical applications, such as the high sensitivity and ultra fast detection of proteins, DNA and ultra bright and photostable metal-enhanced fluorescence based particles for downstream cellular imaging applications. In addition, there are a lot downstream applications of MEP such as in photodynamic therapy by surface plasmon controlled single oxygen generation. Current thinking, describes Metal-Enhanced Fluorescence as the near-field coupling of electronic excited states to surface plasmons (a surface mirror dipole), the particle subsequently radiating the photophysical characteristics of the coupled excited state in the far-field, remarkably, even vibronic structure. In this paper, we communicate our recent findings for metal-fluorophore interactions and our current thinking and progress towards developing a unified metal-fluorophore description.

234-Pos Board B113

Action-Spectra of Electrochromic Voltage-Sensitive Dyes in an Intact Excitable Tissue

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Voltage-sensitive dyes (VSDs) provide a spatially resolved optical read-out of electrical signals in excitable tissues. Several common fluorescent VSDs display electrochromic shifts of their emission spectra, making them suitable candidates for ratiometric measurements of transmembrane voltages. These advantages of VSDs are tempered by tissue-specific shifts to their fluorescence emission. In addition, the optimal electrochromic dye response occurs in wavelength bands distinct from the dye's maximal resting emission. This ''action spectrum'' can undergo tissue-specific shifts, as well.

We have developed a technique for *in-situ* measurements of the action-spectra of VSDs in intact excitable tissues. Fluorescence emission spectra of VSDs during action potential depolarization were obtained within a single sweep of a spectrophotometer equipped with a CCD array detector. To resolve the subtle electrochromic shifts in voltage-induced dye emission, fluorescence emission spectra measured right before and during field-induced action potential depolarization were averaged over about one hundred trials. Removing white noise contributions from the spectrometer's CCD detector/amplifier via low-pass filtering in Fourier space, the action spectra of all dyes could be readily determined.

235-Pos Board B114

Plasmonic Electricity: A Digital form of Metal-Enhanced Fluorescence

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Fluorescence technologies are entrenched in the biosciences today. In nearly all aspects of fluorescence spectroscopy light is focused and collected by a detector which converts the photon flux into a digital signal which is then displayed. To boost optical signatures many groups have shown that the close proximity of fluorescent species to fluorophores, significantly amplifies the fluorescence signatures many fold, a technology recently described as Metal-Enhanced Fluorescence by the Geddes labs[1] However, hidden within these close-range near field fluorophore-metal interactions is an induced plasmonic current, directly proportional to the excitation irradiance and the concentration of the fluorophores present in the near-field, < 20 nm. The current can be read directly, opening up huge opportunities for both the amplification and the *direct detection of fluorescence,* i.e. digital fluorescence, such as in solar energy conversion, digital immunoassays (Figure 1), DNA detection

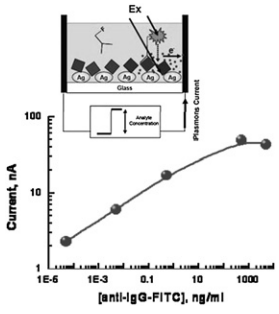

Figure 1. Metal-Enhanced Fluorescence-based digital immunoassays. A model IgG-Anti-IgG assay, demonstrating the direct detection of Fluorescence.