difference between these two worlds is now simply the sample format, i.e., cuvette vs. microplate. It seems reasonable to assume that the cuvette with its straightforward implementation of right angle excitation-emission geometry offers significant advantages over the epi-illumination geometry and uncontained sample imposed by a microplate when it comes to data quality. We have implemented a prototype microplate reader equipped with a variety of pulsed laser sources for measurement of fluorescence spectra, fluorescence lifetimes, and anisotropy. The subject of this poster is benchmarking its performance relative to cuvette format. The plate reader employs direct waveform recording as an alternative to TCSPC; studies to compare the speed, accuracy, and precision of the two lifetime approaches are presented along with several examples of titration curves for rapid determination of binding affinities via time-resolved FRET.

1772-Pos Board B664

Fluorescence Quenching of Tryptophan and Tryptophanyl Dipeptides in Solution

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We report measurements of fluorescence quantum yields of tryptophan, tryptophanylaspartate and tryptophanylarginine in several solvents as well as in aqueous solutions over a wide range of pH. We aim to test a computational model developed by Callis and coworkers [Vivian, J.T. and Callis, P.R. Chem. Phys. Lett. **2002**, *369*, 409] of fluorescence quantum yield, which postulates that quenching in tryptophan arises from energy loss due to an electron transfer from the aromatic system of tryptophan to one of the amides in the protein backbone. Since the electron transfer state is expected to be high in energy, normally this would not be a possible outcome, but because of its large dipole, such a state should be more accessible in polar solvents. In addition, conditions of low (high) pH, which result in a net positive (negative) charge for the backbone should result in an increase (decrease) of electron transfer rates and low (high) quantum yields. The observed results confirm the predictions of the model.

1773-Pos Board B665

Multiscale Diffusion of Single Molecules in Biomimetic Crowding

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Molecular crowding in living cells is believed to influence diffusion processes, intermolecular interactions, protein folding, and intracellular transport. Here, we have investigated crowding effects on the rotational and translation diffusion of Rhodamine green (RhG) and enhanced green fluorescent proteins (EGFP); as compared with homogeneous solvents (buffer and glycerol). Time-resolved fluorescence anisotropy (picoseconds - nanoseconds) and fluorescence correlation spectroscopy (microseconds - seconds) were used to elucidate the effect of non-specific binding on of RhG and EGFP diffusion in synthetic (Ficoll-70, Ficoll-400) and proteins (bovine serum albumin, BSA, and ovalbumin) biomimetic crowding. Using Stocks-Einstein model, the measured rotational-to-translational diffusion coefficient ratios of RhG and EGFP indicate that the non-specific binding and deviation from Brownian diffusion depend on the type of crowding agents. These results provide new insights into crowding effects on diffusion and nonspecific binding of fluorophores on multiple scales of time and concentration.

1774-Pos Board B666

Characterization of Fluorescent Base Analogs to Study DNA Base Flipping at the Single Molecule Level

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Chemical damage to DNA bases can result in mutations, block replication, and lead to cancer. It has been suggested that the phenomenon of base flipping take place by some enzymes during the repair of DNA damages. However, it still remains to be answered if the enzyme ''pushes'' the nucleotide out of the helix (active mechanism) or if the enzyme binds to a provisional flipped base (passive mechanism). Single molecule fluorescence has demonstrated to be a powerful technique to determine the formation of one or more intermediates, and to study the kinetics of the processes from the instant before an enzyme interact with the DNA until the release of the enzymatic product, one molecule at a time. Therefore, in order to optimize and maximize the repair of damaged DNA, new single molecule approaches to fully assess the kinetic mechanism of the base flipping process are needed.

In previous work, the adenine fluorescent base analog 2-aminopurine (2AP) has been extensively used to study base flipping in ensemble average experiments. In addition, a novel 2AP single molecule approach was recently developed.[1] In order to generate single molecule fluorescence assays to probe base flipping in different DNA-enzyme complexes, we need to study fluorescent base analogs (FBA) for all the natural bases. Several FBA molecules have been synthesized during the last four decades and we have selected one FBA molecule for each DNA base to probe base flipping. We have characterized the fluorescent properties of different FBA-substituted DNA molecules that mimic the different states proposed for the base flipping process.

[1]Alemán, E.A., Patrick, E., de Silva, C., Musier-Forsyth, K. & Rueda, D. Single-molecule dynamics with fluorescent nucleotide analogues. *In preparationto be submitted*

1775-Pos Board B667

Temporal Dynamics and FRET Restrained Modeling of an "Invisible" Excited State of T4 Lysozyme

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Conformational fluctuations play a central role in enzyme catalysis. However, extracting a 4D view, i.e. structural changes over time, has represented a big challenge in molecular biophysics. For example, in most cases not all conformers of proteins are visible using the standard structural techniques such as X-ray crystallography or nuclear magnetic resonance. In this work, we present our approach using a fluorescence spectroscopic toolbox to resolve three different conformers of the bacteriophage T4 lysozyme (T4L) and their dynamics. We created a set of more than 20 double mutants specifically labeled with a Förster resonance energy transfer (FRET) pair via the insertion of an unnatural amino acid and a single cysteine. Ensemble time correlated single photon counting (eTCSPC) revealed their corresponding population fractions and provides with structural information. Nevertheless, single molecule FRET, in confocal illumination, showed fluorescence lifetime averaging in timescales faster than diffusion time. To fully characterize the dynamics we used filtered fluorescence correlation spectroscopy which combined with eTCSPC represent a time resolution of seven orders of magnitude (ns to ms). In all, we used the measured distance network to generate a FRET restrained model of the three conformers with high precision. The open conformer appears readily available for substrate binding; the close conformer is very similar to the covalent enzyme-substrate adduct in the T26E mutant of T4L; the third conformer appears more compact than the adduct form which, at present, has not been reported in over more than 440 entries in the protein data bank.

1776-Pos Board B668

Photoblinking and Photobleaching of Single Molecule Fluorescent Probes Induced by Mn^{2+}

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Photophysical phenomena leading to blinking and irreversible photobleaching represent a major obstacle that limits the utility of organic fluorophores in fluorescence-based techniques involving the detection of small numbers of molecules. In recent work, we demonstrated that the coordination of paramagnetic transition metals (e.g. Mn^{2+}) to DNA induces intersystem-crossing in dyes covalently attached to the nucleic acid. This results in fluorescence quenching, triplet blinking and accelerated photobleaching. The increase in triplet formation in the presence of manganese was demonstrated using transient absorption techniques and fluorescence correlation spectroscopy for a series of rhodamine and cyanine dyes, including TAMRA, Cy3 and Cy5. These results are particularly relevant for single-molecule or fluorescence correlation spectroscopy experiments aimed to study enzymes that act on DNA, where Mn^{2+} is used to relax the sequence-specificity of enzymes that catalyze phosphoryl transfer reactions (e.g. polymerases and restriction endonucleases).

1777-Pos Board B669

One and Two Photon Fluorescence Correlation Spectroscopy on Proteins in Glucose Solutions

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Cryopreservation is a powerful technology with many applications in biomedical fields from organ preservation to cellular research. Simple sugars such as glucose and fructose are among the most widely used cryopreserving agents, yet despite their widespread use, the mechanism through which sugars protect

cells on the molecular level is poorly understood. The vitrification model posits that the role of sugars are merely to inhibit ice formation altogether. The water replacement model predicts sugars preferentially bind to the surfaces of protein forming a protective layer against the denaturing ice formation. Under this paradigm, one would expect the hydrodynamic radius of diffusing protein to increase with increasing sugar concentration. In order to test this hypothesis, we have developed both one-photon and two-photon fluorescence correlation spectroscopy (FCS) to measure the hydrodynamic radius of fluorescent particles in glucose solutions. The two-photon technique was developed to investigate tryptophan-containing proteins. However, to date, we have only succeeded in measuring the diffusion of reasonably large, Avidin-coated polystyrene spheres that possess sufficient fluorescence to be reliably detected. Work is continuing on using this technique to study the diffusion of the protein hemocyanin. Given these difficulties with the limited brightness of tryptophan, the one-photon FCS setup is being used to investigate green fluorescent protein (GFP), a much smaller, but considerably brighter, fluorophore.
This work was supported through funding from National Institutes of Health (grant #1R01EB009644-01).

1778-Pos Board B670
Isotropic Resolution in Localization-Based Super-Resolution Microscopy by Single Objective Emission Interference
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The three-dimensional localization of single molecules is a key feature for localization based super-resolution microscopy. Simple methods for molecule localization along the optical axis typically provide a precision that is about 2.5 times worse than lateral. Dual objective microscopes with 4Pi emission detection can improve the axial localization precision to match the lateral. However, 4Pi detection requires complicated instrumentation, intricate maintenance and expensive parts. Therefore, dual objective 4Pi detection is not suitable for everyday biological use. We demonstrate that 4Pi detection for localization based super-resolution microscopy can be realized by using only one objective and a mirror, which eliminates the drawbacks of dual objective 4Pi detection, but still provides isotropic localization precision.

1779-Pos Board B671
FLIM Phasor Analysis for Time-Domain and Frequency-Domain Data
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The phasor analysis of FLIM images provides a fit free global view of molecular species and their interaction in cells and tissues. Different techniques are used to collect the original data either in the time domain or in the frequency domain. The ''phasor transformation'' which is based on the calculation of Fourier components should in principle make the phasor plot independent of the domain of data collection. However, technical differences between the modalities of data acquisition in various instruments result in slightly different phasor calculations. In this poster we discuss the origin of the variations between the different methods of data acquisition. In particular we compare data obtained with the classical analog frequency domain instrument, data obtained with the FLIMbox principle that is based on a digital equivalent of the frequency domain instrument and data obtained with the popular time-correlated single photon counting instrument. We discuss how to minimize these differences which could results in phasors plots that can be directly compared form data obtained with different instruments. We also discuss and compare methods of data filtering which can decrease the noise in the phasor plot without affecting the resolution of FLIM images. Finally we compare phasor plots obtained for different harmonics of the laser repetition frequency. We show that the phasor plot at high harmonics from autofluorescence tissue samples can distinguish between various extracellular components such as the weak fluorescence from collagen and elastin. Work supported in part by NIH-P41 P41-RRO3155, 8P41GM103540 and P50-GM076516

1780-Pos Board B672
Measurements of Fluorescence Decay Times by the Digital Parallel Frequency-Domain Method (FLIMbox)
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The digital parallel frequency domain design is a powerful approach that offers the possibility to implement a variety of different applications in fluorescence spectroscopy and microscopy. It allows lifetime measurements in a cuvette, fluorescence lifetime imaging and FCS as well as multi frequency and multi wavelength tissue imaging in small portable medical devices. It dramatically reduces the acquisition time from the several minutes scale to the seconds scale, and performs every signal process in a digital fashion, avoiding any RF emission and it is extremely inexpensive. This development is the result of a systematic study carried on the previous design known as the FLIMbox. The extensive work done in maximizing the performance of the original FLIMbox led us to develop a new hardware solution with exciting and promising results and potentials that were not possible in the previous hardware realizations. The new design permits acquisition of the full harmonic content of the sample response when it is excited with a pulsed light source in one single measurement using the digital mixing principle that was developed in the original FLIMbox. Furthermore, it is very stable, has very low power requirements and higher precision, and allows the multi exponential analysis. The FLIMbox can be synchronized with lasers that are intrinsically modulated or can generate a frequency to amplitude modulate a laser diode or LED. It provides up to four input channels, it has a saturation feedback control to avoid any time information loss, and it is only limited by the number of photons collected rather than by the sampling window implementation scheme. Work supported by NIGMS, 8P41GM103540.

1781-Pos Board B673
Dual Channel Detection of Ultra Low Concentration of Bacteria in Real Time and via Scanning Fcs
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The rapid quantification and identification of infectious disease agents is of primary importance for medical diagnosis, public health, food safety and environment monitoring. Here we describe an alternative, simple and rapid method to detect very low concentrations of bacteria in water. Our device consists of a small confocal microscope with a horizontal geometry with large pinhole and a holder for cylindrical cuvettes. Two motors provide a rotational and slower vertical inversion motion of the cuvette, so as to scan a total volume of 1ml/min. The device looks like a simplified flow cytometer without flow. Bacteria are stained by two nucleic acid dyes that fluoresce green and red and excited with two lasers. When a bacterium passes through the observation volume emits both red and green fluorescence. The light emitted from the sample is directed by a system of lens toward a dichroic beam splitter and then separated into two light paths for red and green fluorescence detection respectively. Data are analyzed with a correlation filter program based on particle passage pattern recognition. The passage of a particle trough the illumination volume is mimicked with a Gaussian pattern in both channels. The width of the Gaussian correlates with the time of passage of the particle. When the program finds a match with a Gaussian in both channels one particle is counted. The concentration of particles in the sample is deduced from the total number of coincident hits and the total volume scanned. This portable setup provides higher sensitivity (up to few bacteria per ml), rapid results, low cost and a wide use ranging from clinical applications to pollution monitors and water and air quality control.
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1782-Pos Board B674
Measurement of Membrane GPI-GFP Confinement and Dynamics by Image Correlation Spectroscopy
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Current models of the cell membrane assume a heterogenous environment containing domains. Sphinomyelin and cholesterol enriched nano- and micro- domains form a subcategory of membrane heterogeneities, which are thought to functionally sequester proteins. Membrane proteins may also be sequestered by the meshwork membrane proximal actin cytoskeleton. Here we show that image correlation spectroscopy (ICS) based techniques, applied to standard TIRF fluorescence microscopy image series, can be used to characterize these domains. To validate ICS measurements for such systems, we simulated a confined tracer particle diffusion in meshwork and microdomains where we varied the domain size, domain density, confinement probability and diffusion coefficients over ranges reported for cells. We show how one can extract from the correlation function data the characteristic parameters of the system such as apparent domain sizes and characteristic diffusion coefficients. We establish the limits due to the spatio-temporal sampling and noise. Using the simulations, we established the minimum number of domains that need to be sampled by tracer particles for the emergence of confined dynamics features in the correlation function. Finally, we applied this analysis to the study of dynamics of raft associated GPI-GFP in COS-7 cell membranes and verified that our tools can detect changes in the confinement parameters following the application of drugs that disrupt rafts. The recovered large spatial scale diffusion coefficient increased from 0.06 to 0.1 um^2/s when cells were exposed to cholesterol