SNAP-β_2AR by 76 ± 2% ($P<0.001$; $n=4$). Fixation did not change acceptor emission after direct excitation, nor did it change donor emission in the absence of an acceptor. Similar FRET decreases were observed when mobility was decreased by crosslinking with biotin and avidin, or by decreasing temperature. The effect of decreased mobility on FRET was evident for both proteins across a wide range of expression levels. These results suggest that lateral diffusion significantly enhances FRET between membrane proteins. Because diffusion can only enhance FRET between donors and acceptors that diffuse independently (i.e. are not part of the same complex), these results further suggest that a substantial fraction of GPI-anchored proteins and β_2 adrenergic receptors exist on the cell surface as rapidly-diffusing monomers.
Supported by NIH grants GM078319 and GM096762.

3504-Pos Board B659
Applications of Phasor Plots to Dynamic Polarization Studies
Carissa M. Vetromile, David M. Jameson.
University of Hawaii, Honolulu, HI, USA.
The phasor method of treating fluorescence lifetime data provides a facile and convenient approach to characterize lifetime heterogeneity and to detect the presence of excited state reactions such as solvent relaxation and Förster resonance energy transfer. Phasors can be calculated using either frequency domain or time-domain data. The phasor approach has become a valuable tool for both in vitro cuvette studies and in fluorescence lifetime imaging microscopy (FLIM) studies with live cells. In addition to intensity decay, e.g., lifetime data, time-resolved fluorescence can be used to provide information on the rotational mobilites of molecules. This information is acquired in the time-domain using time-decay anisotropy or its frequency domain equivalent known as dynamic polarization. Here, we apply the phasor method to frequency domain dynamic polarization data to provide graphical information on the rotational modalities of fluorophores in vitro. The method is illustrated using a series of simple model systems including fluorophores in isotropic solvents of varying viscosities as well as more complex systems including ethidium bromide interaction with tRNA and also proteins exhibiting varying extents of tryptophan motion. This work was supported in part by funding from Allergan, Inc.

3505-Pos Board B660
Simulations of Fluorescent Probes Attached to SERCA
Bengt Svensson[1], Xiaoqiong Dong[1], Elizabeth L. Lockamy[1], Howard S. Young[2], David D. Thomas[1].
[1]University of Minnesota, Minneapolis, MN, USA, [2]University of Alberta, Edmonton, AB, Canada.
We have performed molecular dynamics simulations of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) labeled with fluorescent probes in order to establish a more rigorous foundation for analyzing fluorescence data from this system. Site-specific labeling of a protein with a fluorescent probe can provide insight into local structural dynamics, based on fluorescence quenching or anisotropy measurements, or based on fluorescence resonance energy transfer (FRET) to another label. To interpret the experimental results from fluorescence spectroscopy in a structural biology context, we have undertaken a combined approach involving X-ray crystallography and computational simulations. To be able to perform these simulations, we developed CHARMM force-field parameters for the fluorescent probes used. Quantum chemistry calculations were used to determine the orientation of the transition dipole moments, which are needed to calculate fluorescence data from the simulations. SERCA was labeled at position Cys674 in the P-domain with the fluorescent probe IAEDANS. A crystal structure of IAEDANS-labeled SERCA was used as a starting point for molecular dynamics simulations of the fluorescent probe and its protein environment. The simulations were each run for 96ns. The transition dipole autocorrelation functions were calculated from the trajectories and were shown to agree with experimental measurements by fluorescence anisotropy. FRET was measured using AEDANS as the donor and TNP-ADP bound in the nucleotide pocket as the acceptor. The interprobe distance R and the orientation factor κ^2, determined from the simulations were used to calculate expected fluorescence lifetime changes due to FRET. The results show that we have established a reliable framework for both fluorescence experiments and simulations in this system. This work was supported by NIH (GM27906, AR007612) and the Minnesota Supercomputing Institute.

3506-Pos Board B661
Determination of Protein Complexes with NADH in Live Cells
Michelle A. Digman, Sohail Jahid, Lucy Chiang, Enrico Gratton.
University of California Irvine, Irvine, CA, USA.
The ratio of free/bound NADH is used to measure the redox state of the cells. This ratio does not provide specific information about which proteins are involved in binding of NADH. In vitro, FRET between tryptophan containing proteins (excited between 280-300 nm) and the NADH co-factor (emitting between 400-450 nm) has been exploited to detect the ratio of free/bound NADH to specific proteins. In live cell microscopy this approach is difficult to implement because it requires UV excitation. If a specific protein interacting with NADH has an absorption or fluorescent moiety, this will result in the quenching of the NADH fluorescence. However, since there is a large amount of NADH, this decrease of fluorescence is difficult to associate to the specific protein. Instead, when FRET occurs between NADH and a fluorescently tagged protein there will be a sensitized emission of the acceptor molecule which is easy to detect. Here we develop an in vivo FLIM/FRET assay in which we use FRET between the NADH (using 2-photon excitation at 740 nm) and the green fluorescent protein attached to an NADH binding protein. In our assay, we use the histone 2B-EGFP construct which is localized in the nucleus and has been shown that this tagged protein does not modify its activity. This FLIM/FRET assay can be used to determine transcription factor binding partners of NADH if the transcription factor is labeled with a fluorescent protein. In addition the FLIM data unequivocally identifies the emission of the GFP from other intrinsic autofluorescent signals (FAD). The phasor lifetime map of histone bound NADH is generated to show areas of increased chromatin activity. NADH is excited in the UV using 2-photon excitation. Thi work is supported in part by NIH-P41 P41-RRO3155, 8P41GM103540 and P50-GM076516.

3507-Pos Board B662
Two Photon Photophysics of Fluorescent Protein Calcium Indicators
John J. Macklin[1], Jasper Akerboom[1], Eric R. Schreiter[1], Lin Tian[1,2], Ronak Patel[1], Jörg Mütze[1,3], Vijay Iyer[1], Bill Karsh[1], Jennifer Colonell[1], Timothy D. Harris[1], Loren L. Looger[1].
[1]HHMI - Janelia Farm Research Campus, Ashburn, VA, USA, [2]Center for Neuroscience, University of California, Davis, CA, USA, [3]Carl Zeiss AG, Jena, Germany.
Fluorescent proteins designed to undergo calcium-dependent changes in emission intensity, when combined with two-photon imaging, have enabled visualization of action potentials and neural activity in awake behaving animals. The two-photon-excited photophysical properties, particularly brightness and photostability, play a critical role in the performance of current indicators and should help guide the design of future indicators. As a benchmark against which to understand and compare these properties *in vivo*, we describe solution measurements on purified proteins from the GCaMP family of calcium indicators. We use Fluorescence Correlation Spectroscopy (FCS) on purified proteins to determine brightness under two-photon excitation, and characterize proteins by their peak brightness. We use emission and two-photon excitation spectroscopy, time-correlated single-photon counting, and FCS to characterize the photophysical properties, and compare photobleaching under fixed-focus steady-state 80 MHz excitation to that of periodic 80 MHz excitation typical of a scanning two-photon microscope.

3508-Pos Board B663
Engineering, Characterization and usage of a Green-To-Red Photoconvertible Dronpa Mutant
Benjamien Moeyaert, Ngan Nguyen Bich, Luc Van Meervelt, Hideaki Mizuno, Peter Dedecker, Johan Hofkens.
KU Leuven, Heverlee, Belgium.
The development of fluorescent proteins (FPs) and recent advances in diffraction-unlimited far-field optical microscopy have truly revolutionized our understanding of life and disease. Especially, genetic fusions of photoactivatable FPs have allowed the visualization of biological events at the nanometer scale. However, for these complicated microscopy schemes, one does not merely need a fluorescing species but rather an intelligent probe. Of remarkable interest are the photoswitchable FPs, that can be reversibly switched off and on, and the photoconvertible FPs that can be irreversibly converted from a green- to a red-emitting state. We previously reported on the rational design of a four-way optical highlighter based on Dendra2, a green-to-red photoconvertible probe (Adam et al, Chemistry & Biology, 2011). This protein, which we called NijiFP, is not only green-to-red photoconvertible, but also shows reversible photoswitching in both states. In the present work, we inverted this question and engineered a similar optical highlighter probe, this time by engineering green-to-red photoconversion properties into the photoswitchable protein Dronpa.
We made ffDronpa, a Dronpa mutant that is formed up to three times as fast as Dronpa, while retaining the interesting photochromic features of Dronpa. Using rational and random mutagenesis, we transformed our fast folding Dronpa to pcDronpa. This Dronpa mutant combines Dronpa's photochromism with the feature of being photoconvertible to a red-emitting state. pcDronpa was studied in detail structurally and spectroscopically and was applied in several microscopic settings.
The addition of pcDronpa to the repertoire of intelligent probes creates new opportunities for protein design and fluorescence imaging. Our example

demonstrates that it is possible to elegantly introduce photodynamic behaviors in a single protein scaffold, in this case Dronpa. This work will open up new possibilities for rational and random design of FPs and their complicated photodynamic properties.

3509-Pos Board B664

Bayesian Fluorescence Correlation Microscopy to Resolve Heterogeneity in Membrane Protein Dynamics

Syuan-Ming Guo[1], Xiaoxiao Ma[2], Nirmalya Bag[2], Nilah Monnier[1], Jun He[1], Thorsten Wohland[2], Mark Bathe[1].
[1]MIT, Cambridge, MA, USA, [2]National University of Singapore, Singapore, Singapore.

Fluorescence Correlation Spectroscopy (FCS) is a powerful tool to measure molecular dynamics with single molecule sensitivity, including local concentrations, aggregation states, and transport mechanisms. Due to its high spatial-temporal resolution and non-invasive nature, FCS has been widely used to probe molecular dynamics in living systems. In contrast to single point detection in traditional confocal FCS, advances in Electron-Multiplying Charge-Coupled Device (EM-CCD) camera technology, Total Internal Reflection Fluorescence Microscopy (TIRFM), and Single-Plane Illumination Microscopy (SPIM) now enable parallel measurements at hundreds to thousands of spatial locations either on cell surface membranes or within tissues. However, objective, automated Multiple Hypothesis Testing (MHT) for such highly parallel FCS measurements is challenging due to sampling limitations, sample heterogeneity, and the lack of automated procedures to accurately estimate the highly correlated noise that is present in Temporal Autocorrelation Functions (TACFs). Thus, automated, unbiased MHT procedures are critical for the proper analysis and interpretation of highly parallel FCS measurements as obtained from TIRFM and SPIM. Here, we apply a Bayesian inference procedure for MHT of competing models for FCS datasets. Simulated FCS measurements demonstrate that the Bayesian procedure selects the simplest model that describes the observed data, thereby capturing heterogeneity in the sample while avoiding over-fitting. Further, model probabilities provide a reliability test for the downstream interpretation of measured parameter values. We apply the procedure to TIRFM FCS data of phase-separated supported lipid bilayers and also Epidermal Growth Factor Receptor (EGFR), demonstrating that membrane and receptor heterogeneity and dynamics are captured when noise is sufficiently low. Our results demonstrate that the Bayesian approach provides an automated, unbiased procedure for FCS data analysis and interpretation with broad applicability to resolving heterogeneous molecular dynamics in biological processes interrogated using TIRFM and SPIM.

3510-Pos Board B665

Modeling Fluorescence Observables, Particularly for FRET Experiments, using Markov Chain Analysis of Molecular Dynamics and Quantum Mechanics Simulations

Amy L. Speelman[1], Aurora Munoz-Losa[2], Katie L. Hinkle[1], Darren B. VanBeek[1], Benedetta Mennucci[2], **Brent P. Krueger**[1].
[1]Hope College, Holland, MI, USA, [2]Università degli Studi di Pisa, Pisa, Italy.

We present a new method for simulating fluorescence observables, particularly those related to bulk and single-molecule fluorescence-detected resonance energy transfer (FRET) experiments. In this method, a molecular dynamics (MD) simulation is used to sample configuration space and quantum mechanics calculations are used to estimate the electronic coupling between the donor and acceptor probes for snapshots along the MD trajectory. A Markov chain method is used to sample the resulting electronic coupling trajectory allowing accurate simulation of any desired fluorescence observables, such as single-molecule FRET efficiency histograms or time-resolved donor fluorescence decays. The Markov chain results will be compared with the results of simple histogram and averaging schemes showing that the Markov chain is the only one that yields realistic results in well known examples such as the rapid diffusion limit. This combination of computational methods also avoids some pitfalls of traditional FRET analysis such as the kappa-squared and the ideal dipole approximations. Because the simulation results can be compared directly with experimental observables, this method may allow more detail to be derived from experiment than is traditionally possible.

3511-Pos Board B666

A Microplate-Based Instrument for Resolving Fluorescence Lifetimes

Karl J. Petersen[1], Sutton E. Higgins[2], Kurt C. Peterson[1], Gregory D. Gillispie[1], David D. Thomas[1].
[1]University of Minnesota, Minneapolis, MN, USA, [2]Dartmouth College, Hanover, NH, USA.

We describe a microplate-based, time-resolved fluorescence (TRF) spectrometer, able to detect multiple fluorescence lifetimes with acquisition times comparable to existing steady-state plate readers. The instrument combines high-energy pulsed laser sources (10-20 kHz repetition rate, 1-3 ns pulse width) with a photomultiplier and high-speed digitizer (1 GHz) to record a complete fluorescence decay waveform after each pulse. Pulse-averaged measurements of dyes in single wells yielded lifetimes comparable in accuracy and precision to single photon counting (SPC.) Lifetimes as short as 1 ns may be resolved by interleaving with an effective sample rate of 5 GHz. In a multiple-well format (96- or 384-well plates) the response of a dye to concentration gradients was easily observed, e.g., in quenching by acrylamide. The precision was sufficient to resolve mole fractions in mixtures of single-exponential dyes with an acquisition time of 0.5 s per well. In multiple-well experiments the variation in total measured fluorescence was comparable to steady-state instruments, while the precision in lifetime was better than 2%. These features will enable rapid, multiple-well, time-resolved FRET and fluorescence quenching experiments to determine changes of structural equilibria in solution, cells, and reconstituted systems.

3512-Pos Board B667

Time Resolved Fluorescence Studies of Genetically Encoded Protein Sensor Frex for Nadh and pH Levels

Yuting Chen[1], Sanjun Zhang[1], Rongrong Liang[1], Mengfang Chang[1], Haifeng Pan[1], Yuzheng Zhao[2], Yi Yang[2], Jianhua Xu[1].
[1]East China Normal University, shanghai, China, [2]East China University of Science and Technology, shanghai, China.

Reduced nicotinamide adenine dinucleotide (NADH) and its oxidized form, NAD^+, are the most important coenzymes found in all living cells. For many years, researchers have relied on the weak NADH endogenous fluorescence signal to determine the NADH level. We have recently reported a series of genetically encoded fluorescent sensors highly specific for NADH level[1]. However, those NADH sensors are also found to be very sensitive to pH value of the local environments, which may result in errors in the analysis of NADH level or NADH/NAD^+. We here report that the time resolved fluorescence of NADH sensor Frex can simultaneously characterize the pH and NADH level by using a time correlated single photon counting (TCSPC) technique, whose scale is from 100ps to 30ns. Frex sensors were excited at 420 and 485 nm, respectively. Both the total fluorescence photon counting number *N* and the fluorescence time distribution have been obtained. All fluorescence transients of Frex sensors reveal complex, i.e. multiexponential behavior. Similar to our previous report[1], the *N* ratio under two excitation wavelengths N_{485}/N_{420} can be used to characterize the NADH level. Meanwhile, the decay associated spectra (DAS) can be fitted by 3 exponential components decay function. We found that the percentage α of the components with the lifetimes of about 0.2, 1 and 3 ns were proportional to the pH values. Therefore, both the NADH and pH levels can be obtained from time resolved fluorescence spectra. No parallel experiment is needed any more to calibrate the pH effects. Candidate mechanisms and further experiments including femtosecond dynamics will be discussed.

Reference:
1 Zhao, Y. *et al.* Genetically Encoded Fluorescent Sensors for Intracellular NADH Detection. *Cell Metab***14**, 555-566, (2011).

3513-Pos Board B668

Improving FRET Dynamic Range with Bright Green and Red Fluorescent Proteins

Amy J. Lam[1], François St-Pierre[1], Yiyang Gong[1], Jesse D. Marshall[1], Michael R. McKeown[2], Mark J. Schnitzer[1], Roger Y. Tsien[2], Michael Z. Lin[1].
[1]Stanford University, Stanford, CA, USA, [2]University of California, San Diego, San Diego, CA, USA.

FRET between fluorescent proteins is widely used to monitor biochemical processes in living cells as the length scale of FRET is well matched to protein conformational changes and protein-protein interactions. Although most FRET-based reporters rely on CFPs and YFPs, aspects of CFPs and YFPs are problematic for FRET. CFPs and YFPs can undergo rapid multirate and reversible photobleaching, YFPs can photoconvert into cyan fluorescent species, CFPs can photoactivate at YFP excitation wavelengths and the violet CFP excitation light can be phototoxic. Furthermore, many CFP- and YFP-based FRET reporters produce small changes in FRET, creating detection challenges when the imaged structures are small or when biochemical responses are subtle or transient.

In this work, we have developed new fluorescent proteins with properties better suited to a wide range of FRET applications. Starting from Aequorea victoria GFP and the RFP mRuby, we engineered Clover and mRuby2, which conferred greater dynamic range and photostability to four existing FRET reporter designs. Among the improved reporters are a voltage sensor that allows more reliable detection of single action potentials than do previous sensors and an improved RhoA reporter able to detect local and rapid RhoA activation in neuronal growth cones during ephrinA-stimulated retraction.