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.
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3504-Pos Board B659
Applications of Phasor Plots to Dynamic Polarization Studies
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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
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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
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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
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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
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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