3897-Pos
Application of Phasor Plots to Analysis of Fluorophore Heterogeneity, Excited State Reactions and Protein Conformations
Nicholas G. James[1], Martin Štefl[2], Justin A. Ross[1], David M. Jameson[1].
[1]University of Hawaii, Honolulu, HI, USA, [2]Academy of Sciences of the Czech Republic, J. Heyrovsky Institute of Physical Chemistry, Prague, Czech Republic.
Phasor plots provide a simple graphical method to visualize and quantify time resolved fluorescence data, obtained using either frequency or time domain methods, independent of model constraints. Using the phase and modulation approach, the phasor plot converts raw data at a single frequency to a vector. Single exponential decays appear on the universal circle (semicircle with radius 0.5 and center 0.5, 0) whereas decays due to multiple exponentials appear as points inside the universal circle. This method has been successfully applied to fluorescence lifetime imaging microscopy (FLIM) wherein the data are typically collected at only one frequency. Applications of phasor plots in FLIM studies have, to date, been largely limited to FRET studies in cells. We have extended the application of phasor plots to several in vitro systems. Specifically, we have analyzed frequency-domain data of binary and tertiary mixtures of non-interacting, monoexponential-decay fluorophores and intrinsic protein fluorescence using the phasor plot approach. Phasor points from binary mixtures of varying composition lie along the line connecting the individual component points on the universal circle, while tertiary mixture points fall in a triangle between the individual vectors. Molecular interactions such as protein dissociation, protein-ligand interaction, denaturation, and energy transfer resulted in changes in the position of the vector point allowing for a rapid, graphical representation of these complex reactions. Data at a single frequency may be recorded rapidly allowing resolution of kinetic processes that would be difficult to monitor using complete multifrequency approaches. The combined results demonstrate the value of the phasor plot method to in vitro lifetime analysis. This work was supported in part by a grant from Allergan, Inc.

3898-Pos
Receptor-Ligand Interactions in the Plasma Membrane of Live Cells Resolved in Space and Time by N&B Analysis
Christian Hellriegel[1], Valeria R. Caiolfa[2,1], Nicolai Sidenius[3], Enrico Gratton[4], Moreno Zamai[2,1].
[1]CNIC, Madrid, Spain, [2]San Raffaele Scientific Institute, Milano, Italy, [3]IFOM- Italian Cancer Research Foundation FIRC, Milano, Italy, [4]Laboratory for Fluorescence Dynamics, University of California Irvine, Irvine, CA, USA.
In this presentation we show how we push the Number and Brightness analysis (N&B) to the limits of applicability. We demonstrate that by N&B we can observe how a GFP labeled membrane receptor (namely uPAR) dimerizes upon ligand binding in live cells. We show how we obtain real time, spatially and temporally resolved images of the molecular reorganization of uPAR in the cell membrane. These results are backed by extensive simulations, and by well-defined live cell calibration experiments (using monomeric and dimeric GFP-uPAR constructs).
N&B quantifies the amplitudes of fluorescence intensity fluctuations as individual fluorescent species diffuse in and out of a pixel in a series of images. The basic idea is that the amplitude fluctuations of a diffusing molecule labeled with two dyes (e.g. a dimer, or a bound ligand-receptor pair) will be twice as large as the amplitudes of a molecule with only one dye (i.e. a monomer, or the unbound ligands and receptors), simply because the doubly labeled object is twice as bright as the individual one.
N&B is related to fluctuation spectroscopy such as fluorescence correlation spectroscopy FCS and photon counting histogram, PCH. These methods can resolve molecule-molecule interactions, but are usually restricted to the acquisition at one specific pixel. N&B was described recently for 2-photon scanning microscopy. There, N&B was typically used to distinguish between mobile molecules and large aggregates in cells, using time-sequences of about 50-100 frames (typically 512x512 pixels at 4s/frame).
However, when attempting to distinguish between monomers and dimers, as the smallest possible increment of molecule-molecule interactions, the experimenter is confronted with low signal-to-noise ratios and long-term perturbations (cell movement, vesicle trafficking). In this work we describe how we have resolved these issues.

3899-Pos
Anomalous Diffusion as a Readout for the Folding Status of Transmembrane Proteins
Nina Malchus, Laura Weimann, Matthias Weiss.
German Cancer Research Center, Heidelberg, Germany.
A multitude of transmembrane proteins enter the endoplasmic reticulum (ER) as unfolded polypeptide chains. In the ER, chaperones supervise their folding process and prevent, by still poorly understood mechanisms, a premature export from the ER.
Here, we used Fluorescence Correlation Spectroscopy (FCS) to investigate the interaction of a prototypical transmembrane cargo protein, tsO-45-G, a temperature-sensitive mutant of VSV-G, with the ER quality control machinery in vivo by quantifying the proteins diffusion properties in the ER under various conditions. Our experimental data and accompanying simulations show that the diffusion of unfolded tsO-45-GFP in the ER is strongly anomalous, most likely due to a transient oligomerization with UDP-glucose:glycoprotein glucosyltransferase (UGT1). In contrast, folded tsO-45-G, calnexin-associated unfolded tsO-45-G, or a mutant tsO-45-G with only one glycan are significantly less obstructed in their diffusion behavior.

3900-Pos
Counting Up the Molecules in Live Bacillus Subtilis by Fluctuation Imaging and Analysis: An in Vivo Study of Transcriptional Regulation
Matthew L. Ferguson[1], Matthieu Jules[2], Dominique Le Coq[2], Stéphane Aymerich[2], Nathalie Declerck[1], Catherine A. Royer[1].
[1]Centre de Biochimie Structurale, Université Montpellier 1&2, CNRS (UMR 5048), INSERM (U554), 29 rue de Navacelles, F-34090, Montpellier, France, [2]Microbiologie et Génétique Moléculaire, INRA (UMR1238) and CNRS (UMR2585), Agro-ParisTech, F-78850, Thiverval-Grignon, France.
Number & Brightness analysis (N&B) is a useful technique for characterizing the brightness and concentration of fluorescent molecules in vivo. We are interested in regulation networks of the Central Carbon Metabolism of B. subtilis. Here we investigate promoter activity in bacterial strains expressing GFP reporter proteins under control of native promoters engineered into the B. subtilis chromosome. Of particular interest in this study are promoters implicated in the switch between glycolysis and gluconeogenesis. We utilize two photon N&B to monitor the number of GFP molecules within living cells of Bacillus subtilis by quantifying intensity fluctuations from fluorescent images of cells taken on timescales faster than the diffusion of GFP. As expected, changes in promoter activity were dependent upon carbon source and inducer concentration.

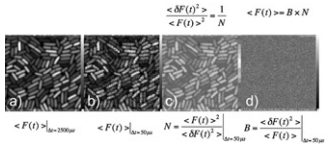

Figure: a) Fluorescence image of B. subtilis cells expressing GFP. b) Image taken with a laser dwell time faster than the diffusion time of a GFP molecule. c) Number map determined from fluctuations at each pixel from 50 fast images. Color scale is from 0 to 18 molecules. d) Brightness map determined from (a) divided by (c).

3901-Pos
Automated Screen of in Vivo Molecular Interactions using Fluorescence Cross-Correlation Spectroscopy (FCCS)
William A. Marshall, Christopher Wood, Jay R. Unruh, John T. Dirnberger, Hans-Martin Herz, Winfried Wiegraebe.
Stowers Institute for Medical Research, Kansas City, MO, USA.
We have developed an automated fluorescence cross-correlation screen to detect molecular interactions in yeast. Using fluorescence cross-correlation spectroscopy (FCCS) positive control strains containing linked eGFP and mCherry proteins could be reproducibly distinguished from negative controls with independently diffusing populations of the two fluorophores. Transmitted light images were acquired in parallel with FCCS measurements to determine protein localization and cell health. Data was taken in a 96-well format on a commercially available microscope with the addition of a ConfoCor3 (Carl Zeiss Jena GmbH, Germany). Custom software controlled navigation of the 96-well plates, detection of yeast cells, and selection of cellular regions for taking FCCS measurements, making the screen adaptable for larger scale experiments. Using this method, proteins of particular interest can be rapidly screened against large portions of the proteome to uncover their contribution to the function of a complex network of proteins.

3902-Pos
Application of Fluorescence Correlation Spectroscopy to Measure High-Density Lipoprotein (HDL) Metabolism
Russell Deitrick, Hamid Razzaghi, Emily A. Gibson.
University of Colorado Denver, Denver, CO, USA.
High-density lipoprotein (HDL) protects vascular system from atherosclerosis by several mechanisms, including reverse cholesterol and therefore understanding its metabolism has important implications for public health. A major factor in HDL metabolism is endothelial lipase (EL). The goal of this study was to elucidate the kinetics of EL-HDL metabolism using fluorescence correlation