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.

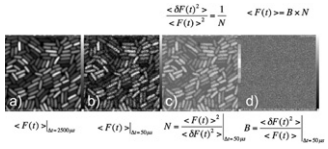

Figure: a) Fluorescence image of B. subtilis cells expressing GFP. b) Image taken with a laser dwell time faster than the diffusion time of a GFP molecule. c) Number map determined from fluctuations at each pixel from 50 fast images. Color scale is from 0 to 18 molecules. d) Brightness map determined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

spectroscopy (FCS). FCS is an advanced microscopy technique in which fluctuations in the fluorescence of a dye or dye-labeled molecule is recorded as the particles freely diffuse through a small focal volume. In this case, we measured the fluorescence of dye-labeled (Nile Red) HDL in the presence of wild type and mutant EL. The data can be analyzed mathematically using the cross-correlation function, from which the diffusion coefficient of the molecule is obtained. The lipase activity of EL changes HDL size, in turn affecting the diffusion coefficient, and can be calculated using the Stokes-Einstein relation. Our preliminary results suggest the hydrolysis of HDL occurs rapidly and proportionately to the concentration of EL. Several mutations in EL have been identified in human population studies. Our future goal is to compare the rate of hydrolysis between wild type and mutant EL and with normal and oxidized HDL. In addition, fluorescence measurements were used to investigate the composition of HDL. The fluorescence spectrum of Nile Red is dependent upon the local lipid environment. By monitoring the change in fluorescence emission as a function of EL metabolism, we investigate the content of HDL while being remodeled by EL. These fluorescence techniques allow us to answer some of the key questions regarding the HDL lipid collection and distribution function.

3903-Pos

In Vivo Imaging of Single-Molecule Translocation through Nuclear Pore Complexes by Pair Correlation Functions

Francesco Cardarelli, Enrico Gratton.
University of California, Irvine, CA, USA.

Nuclear pore complexes (NPCs) mediate bidirectional transport of proteins, RNAs, and ribonucleoproteins across the double-membrane nuclear envelope. We recently introduced a method based on pair correlation functions (pCF) which measure the time the same molecule takes to migrate from one location to another within the cell (1). The spatial and temporal correlation among two arbitrary points in the cell can provide a map of molecular transport, and also highlight the presence of barriers to diffusion with very high time resolution (in the microsecond scale) and spatial resolution (limited by diffraction).

Here we report the use of this method to directly monitor a model protein substrate undergoing transport through NPCs in living cells, a biological problem in which SPT has given results that cannot be confirmed by traditional FCS measurements because of the lack of spatial resolution. Our substrate is composed by a GFP linked to a functional nuclear localization sequence (NLS) and transfected into living CHO-K1 cells: the recombinant NLS-GFP protein can bind to molecular carriers mediating cytoplasm-to-nucleus active import as well as shuttle across the NPC by passive diffusion (its molecular weight is below the cut-off size limit of the NPC).

We show that obstacles to molecular flow can be detected and that the pCF algorithm can recognize the heterogeneity of NLS-GFP intracompartment diffusion as well as the presence of barriers to its transport between compartments (i.e. the NPCs of the nuclear envelope).

(1) Digman, M.A., and Gratton, E. Imaging Barriers to Diffusion by Pair Correlation Functions . Biophys. J. 97, 665-673 (2009).

Work supported in part by NIH-P41 P41-RRO3155 and P50-GM076516.

3904-Pos

Fluorescence Correlation Spectroscopy for Clinical Testing in Von Willebrand Disease

Richard Torres, Michael Levene.
Yale University, New Haven, CT, USA.

The von Willebrand factor (vWF) protein is an essential component of normal coagulation that is present in human plasma as a distribution of multimers composed of 2 to 40 or more monomers. Defects in the synthesis and metabolism of vWF represent the most common inherited abnormalities of coagulation and can be categorized as type 1 for quantitative deficiencies and type 2 for qualitative deficiencies. Current clinical methods for diagnosis and classification of von Willebrand disease suffer from significant limitations relating to the vast number of mutations that can occur, the fact that multimer size is a critical determinant of functional capacity, and the poor reproducibility of available activity assays. We have successfully employed the use of fluorescence correlation spectroscopy (FCS) to address the drawbacks of presently available vWF analysis methods. Autocorrelation curves from fluorescently tagged anti-vWF antibody incubated with plasma from normal donors and controls differ significantly from those obtained with plasma from patients with von Willebrand disease. Furthermore, it was possible to separate type 2 vWD patients from type 1 vWD patients on the basis of the shape and average diffusion time of the FCS curves. Cluster analysis yielded the expected separation of groups based on differences in the amount of antibody bound to antigen and the average diffusion time of bound antibody. Further analysis using a maximum entropy method FCS fitting program (MEMFCS) suggests further subclassification is possible with fluctuation analysis. The results indicate FCS is a practical tool for clinical evaluation of coagulopathic patients suspected of having von Willebrand disease. This research presents one of the first implementations of FCS in analysis of clinical samples.

3905-Pos

Dynamic Imaging and Fluctuation Spectroscopy on Single Microvilli in Opossum Kidney Cells by the Modulation Tracking Method

Luca Lanzano[1], Peter Fwu[1], Hector Giral[2], Moshe Levi[2], Enrico Gratton[1].
[1]Univ California, Irvine, CA, USA, [2]University of Colorado, Denver, CO, USA.

Regulation of renal tubular inorganic phosphate (Pi) transport occurs via the proximal tubular apical brush border membrane (BBM) sodium gradient-dependent Pi (NaPi) cotransport proteins. Distinct families of NaPi cotransporters show differential regulation under dietary and hormonal stimuli, but the way this is accomplished, for instance through localization in distinct BBM micro- or nano-domains and/or preferential interaction with different PDZ proteins, is not yet understood.

Crucial information could come from the application of single molecule fluctuation correlation spectroscopies on the BBM of living cultured Opossum Kidney (OK) cells expressing NaPi co-transporters with different GFP constructs. The BBM is composed of many microvilli, several micron long structures with a diameter of about 100nm. The microvilli show a relatively fast motion (in the seconds time scale) that makes the use of fluctuation spectroscopy difficult.

None of the current nano-resolution optical methods seems capable of measuring the clustering dynamics of proteins on the surface of rapidly moving microvilli. We developed an optical imaging technique called Modulation Tracking (MT) in which we track the center of mass of the microvillus at an arbitrary point along its length while the laser spot rapidly oscillates perpendicularly to the surface and the changes in the modulation are used to measure the distance of the spot from the fluorescent surface with nanometer resolution. High resolution images of the microvilli can be obtained scanning slowly along the microvillus axis. Since the moving microvillus is always at the center of the orbit, fluorescence image correlation techniques can be applied making the MT a truly dynamic nano-imaging technique.

Work supported in part by NIH RO1 DK066029-01A2 (ML, EG), NIH-P41 P41-RRO3155 (EG, PF and LL) and P50-GM076516 (EG, PF and LL).

3906-Pos

Regulation of CFTR on the Plasma Membrane

Asmahan Abu-Arish, Ian R. Bates, Elvis Pandzic, Angela Ho,
John W. Hanrahan, Paul W. Wiseman.
McGill University, Montreal, QC, Canada.

The cystic fibrosis transmembrane conductance regulator (CFTR) is an anion channel which is tightly regulated by phosphorylation and interactions with a macromolecular complex that mediates spatially localized signaling mechanisms. The complex may include scaffolds such as NHERF1, the adaptors ezrin and Receptor for Activated C Kinase (RACK1), and enzymes such as adenylyl cyclase, kinases, phosphatases, and phosphodiesterases. NHERF1 anchors CFTR to the actin cytoskeleton whereas RACK1 mediates its association with protein kinase C (PKC), however the relationships between these proteins remain poorly understood. We have studied the dynamics of fluorescent fusion proteins containing CFTR, NHERF1 and RACK1 using quantitative fluorescence fluctuation imaging techniques. Lateral diffusion coefficients and immobility fractions at the plasma membrane were calculated from time-series of confocal images using temporal image correlation spectroscopy (TICS). We also developed a novel cross-correlation TICS (CC-TICS) analysis for studying the dynamics of interacting protein species and their binding ratios, so that the assembly and disassembly of the CFTR regulatory complex could be studied quantitatively. Initial results indicate that the lateral mobilities of RACK1 ($D=1.5\pm0.6\ \xi10^{-3}\ \mu m^2/s$) and NHERF1 ($D=2.6\pm1\ \xi10^{-4}\ \mu m^2/s$) are both significantly reduced ($D=8.6\pm2.5\ \xi10^{-4}$ and $1.8\pm0.8\ \xi10^{-4}\ \mu m^2/s$, respectively) when co-expressed with CFTR and are further reduced upon activation of PKC ($D=6.6\pm2.2\ \xi10^{-4}$ and $1\pm0.4\ \xi10^{-4}\ \mu m^2/s$, respectively). The fractions of immobility significantly increased whenever the diffusion coefficient decreased. The results suggest two distinct phases during CFTR complex formation; initial tethering under basal conditions followed by aggregation into complexes during PKC stimulation. These preliminary results provide new insight into protein-protein interactions that regulate CFTR, information that is essential for understanding anion transport in cystic fibrosis and secretory diarrhea.

3907-Pos

Predicting Protein Co-Expression Fractions in Living Cells

Elizabeth M. Smith, Yan Chen, Joachim Mueller.
University of Minnesota, Minneapolis, MN, USA.

Fluorescence fluctuation spectroscopy utilizes the fluctuation in a fluorescent signal to determine molecular brightness, concentration, and diffusion properties of fluorescent particles passing through an optical volume. Brightness analysis is