stimulation. We recently demonstrated the phasor approach to biosensor FRET detection by FLIM as a method that is robust towards biosensor design (single and dual chain) as well as the fluorescence artifacts inherent to the cellular environment. Using a frame mode acquisition we were able to map the spatial localization and quantify the fractional contribution of the free and bound state of a dual chain biosensor or the low and high FRET species of a single chain biosensor in each pixel of an image. To increase temporal resolution we find that line acquisition of FLIM data increases the total pixel integration and allows us to probe millisecond to second dynamics of RhoA and Rac1 activity across the cell. Given that this timescale is comparable to the diffusive rate at which Rac1 and RhoA traverse the cell upon activation we concomitantly perform pair correlation function (pCF) analysis along the line scan and investigate the molecular flow pattern of RhoA and Rac1 upon growth factor stimulation. We find for RhoA and Rac1 there are distinct gradients of activation from back to front (FLIM data) and a molecular flow pattern (pCF) that explains the observed polarized GTPase activity.

998-Pos   Board B784
Numerical Methods for Improving the Reliability of Number and Brightness (N&B) Analysis
Antonio Trullo[1,2], Valeria Rosaria Caiolfa[1,2], Moreno Zamai[1,2].
[1]San Raffaele Scientific Institute, Milan, Italy, [2]Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain.
N&B is a technique based on moment-analysis for the measurement of the average number of molecules and brightness in each pixel in fluorescence correlation microscopy images. The average brightness of the particle is obtained from the ratio of the variance to the average intensity at each pixel (Digman et al., 2008).
N&B is useful for determining stoichiometry and oligomerization of protein complexes in live cells. However, the signal is generally affected by some long-term fluctuations, mainly due to cellular motion and photobleaching. These effects lead to an overestimation of variance, and consequently of brightness.
In this work we present a protocol for correcting N&B analysis for these sources of extra-variance.
We sort out the errors due to translational motion by realigning the images of the time series (~250 frames) using a simple routine based on correlation arguments. This correction is useful because N&B is a pixel-based technique. After realignment, a given region of the cell can be associated to each pixel, avoiding signal fluctuations due to the displacement of the cell.
Moreover, we use a particular high-pass filter, the 'boxcar' filter, to correct for the extra-variance introduced by photobleaching, which is an exponential reduction of the response of the fluorophore to laser excitation. Since photobleaching is negligible within a short time period (i.e., a small data segment), the boxcar filter allows the computation of brightness in a sliding segment, spanning all over the signal. The final result is the average over the total collected frames of the brightness values computed for each segment (Hellriegel et al, 2011).
We demonstrate the efficiency of these corrections using simulations of membrane motion and photobleaching. We also provide examples of correction applied to data acquired on EGFP constructs expressed in live cells.

999-Pos   Board B785
A Novel FRET Biosensor For Measuring Glycolytic Activity: A Study of Pancreatic Beta-Cells
Matthew J. Merrins, Leslie S. Satin.
University of Michigan, Ann Arbor, MI, USA.
Pulses of insulin from pancreatic beta-cells help maintain blood glucose in a narrow range, although the source of these pulses is unclear. We propose that a positive feedback circuit exists within the glycolytic pathway employing the allosteric enzyme phosphofructokinase-1 (PFK1), which endows beta-cells with the ability to generate oscillations in metabolism via autocatalytic activation by its product Fructose-1,6-bisphosphate (Fru1,6-BP). To test this hypothesis, we have engineered a family of inter- and intramolecular Cerulean/Citrine FRET biosensors based on the glycolytic enzyme pyruvate kinase M2 (PK), which is allosterically activated and reported to multimerize upon binding Fru1,6-BP. When introduced into Min6 beta-cells, intramolecular PK biosensor apparent FRET efficiency increased dose-dependently in response to glucose (0, 2.5, 11, and 25 mM). This change was rapid (within seconds) and reversible. When Min6 cells were stimulated with 25 mM glucose/TEA, oscillations in PK biosensor activity were evident as ratiometric FRET changes, and exhibited a similar period to slow oscillations in intracellular calcium or NAD(P)H (3-4 min). Our results suggest that glycolysis in beta-cells is oscillatory and that PFK1 is indeed an attractive candidate for the oscillatory generator. More broadly, this family of PK biosensor constructs could be useful for exploring the magnitude and kinetics of glycolytic activity in living cells. Supported by F32DK085960 (M.J.M.) and R01DK46409 (L.S.).

1000-Pos   Board B786
Time-Resolved Confocal Fluorescence Microscopy: A Generalized Approach Enables New Directions for FLIM, FRET and FCS
Samantha Fore[1], Felix Koberling[2], Marcelle Koenig[2], Peter Kapusta[2], Bendedikt Kraemer[2], Benjamin Ewers[2], Rainer Erdmann[2], Steffen Ruettinger[2], Julie L. Fiore[3], David Nesbitt[3].
[1]PicoQuant Photonics North America Inc., Westfield, MA, USA,
[2]PicoQuant GmbH, Berlin, Germany, [3]JILA, NIST and University of Colorado, Boulder, CO, USA.
Fluorescence dynamics of single molecules can be followed on timescales from sub-nanoseconds to seconds and even beyond with a universal approach of time-resolved measurements. The underlying technique (Time-Tagged Time-Resolved (TTTR) Recording) allows one to simultaneously record timing and fluorescence intensity information, both spectrally and spatially, on a single photon basis. We apply photon sorting and weighting schemes determined from the nanosecond photon arrival times to extend and improve single-molecule fluorescence methodologies which up to now commonly utilize only intensity-based analysis, namely FCS and FRET.
In Fluorescence Lifetime Correlation Spectroscopy (FLCS) photon weighting provides superior suppression of common parasitic contributions, e.g., Raman scattering and detector after-pulsing. Beyond this improvement of traditional FCS, FLCS also offers the possibility to accurately determine diffusion properties of different species only requiring that the species differ in their fluorescence lifetimes [1]. In 2-focus-FCS (2fFCS), the nanosecond timing information is used to identify the spatial origin of the photons by combining Pulsed Interleaved Excitation (PIE) with time-gated detection [2]. Thereby, 2fFCS dramatically improves the accuracy of measuring absolute diffusion coefficients. In addition to this, PIE can be used to identify artifacts and subpopulations in single-pair FRET measurements. Nanosecond time-resolved detection offers a complementary approach to donor/acceptor intensity based methods for calculating FRET efficiencies via quenching of the donor lifetime.
[1] Benda A., Hof. M., Wahl M., Patting M., Erdmann R., Kapusta P., Rev. Sci. Instr., Vol.76, 033106 (2005)
[2] Dertinger Th., Pacheco V., von der Hocht I., Hartmann R., Gregor I., Enderlein J, ChemPhysChem, Vol.8, p.433 (2007)

1001-Pos   Board B787
Ultra-Deep Imaging with Cellular Resolution: Enhanced Two-Photon Fluorescence Microscopy with the Use of a Wide Area Photodetector
Viera Crosignani, Alexander Dvornikov, Enrico Gratton.
Univesity of California, Irvine, Irvine, CA, USA.
We have previously shown that the use of a wide photocathode area PMT as a detector in a two-photon fluorescence microscope allowed us to image in turbid samples up to the depth of about 2.5 mm with cellular resolution. This detection scheme enables a very efficient collection of fluorescence photons directly from the wide (1'' diameter) area of the sample, which considerably increases the detection system sensitivity in comparison to a traditional two-photon microscope, where fluorescence is collected by the same objective lens used for excitation. Because the imaging depth depends on the ability of the system to sense weak fluorescent signals, this new detection method significantly enhances the imaging depth. We have recently built a new experimental system that works in the upright configuration, which is best suited for experiments on live animals. The system employs a high power Ti:Sa Mai Tai laser with a group velocity dispersion compensator (DeepSee) for two-photon fluorescence excitation that allows us to extend the imaging depth to 3mm in samples simulating brain tissue optical properties. Imaging experiments in vivo and in vitro have also been conducted on live animals (mice) and tissues (skin, colon, small intestine).
With the aid of the new high speed response PMT that we are currently incorporating in the system, we will be able to perform fluorescence lifetime imaging microscopy (FLIM) on whole animals and tissue samples at a few mm depth. This double feature will particularly aid in vivo neuron imaging.
This work was supported by National Institutes of Health grants: P41-RRO3155, P50-GM076516

1002-Pos   Board B788
Multi-Confocal Fluorescence Correlation Spectroscopy: A Technique for Parallel Multi-Spot Measurements in Living Cells and its Application to the Study of Cellular Response to Heat Shock
Meike Kloster-Landsberg[1], Gaëtan Herbomel[2], Yves Usson[3], Irène Wang[1], Claire Vourc'h[2], Catherine Souchier[2], Antoine Delon[1].
[1]Université Joseph Fourier / CNRS, LIPhy UMR5588, Saint Martin d'Hères, France, [2]Université Joseph Fourier / INSERM, IAB CRI U823 team 10, Grenoble, France, [3]Université Joseph Fourier / CNRS, TIMC (IN3S), Grenoble, France.

We present a novel multi-confocal Fluorescence Correlation Spectroscopy (mFCS) technique that allows simultaneous FCS measurements in different locations within a cell. Standard FCS experiments are usually limited to one observation volume, so that information can only be obtained from one position at a time. In contrast, mFCS makes it possible not only to monitor fast temporal and spatial changes in the dynamics of cellular proteins, but also to increase the amount of data collected per measurement and thus reducing the time necessary to produce statistically significant results. Our mFCS technique takes advantage of a Spatial Light Modulator (SLM) to create several distinct observation volumes at a time. Parallel detection is performed using an Electron-multiplied CCD camera, where pixels act as pinholes for confocal detection. We were able to show that the spatial resolution and the sensibility of our mFCS system is close to that of an classical FCS setup. Employing a special camera readout mode, a temporal resolution of 14 μs is reached, which is adapted to the dynamics of most cellular proteins. The mFCS technique is applied to study the cellular response to thermal stress, by monitoring Heat Shock transcription Factor 1 (HSF1), which is a key regulator of heat shock response. Conducting experiments on living cells, we observed clear changes in the dynamics of HSF1 when heat shocking: its diffusion slows down, together with an increase in the bound fraction and in the residence time.

1003-Pos   Board B789
Real-Time Tracking of Lanthanide Ion Doped Upconverting Nanoparticles in Living Cells

Kang Taek Lee[1], Sang Hwan Nam[1], Yun Mi Bae[1,2], Yong Il Park[3], Jeong Hyun Kim[3], Hyung Min Kim[1], Joon Sig Choi[2], Taeghwan Hyeon[3], Yung Doug Suh[1].
[1]Korea Research Institute of Chemical Technology (KRICT), Daejeon, Korea, Republic of, [2]Chungnam National University, Daejeon, Korea, Republic of, [3]Seoul National University, Seoul, Korea, Republic of.
Lanthanide ion-doped upconverting nanoparticles (UCNPs), which emit in the visible range upon absorption of NIR photons, have attracted great attention in the area of biological imaging owing to their unique properties. First, two-photon upconversion of NIR excitation to the emission of a visible photon is so efficient that a tiny CW laser with the output of tens of milliwatts is sufficient as the excitation source. Second, by employing NIR excitation, one can suppress cellular autofluorescence, hardly induce photodamage to cells, and achieve relatively deep penetration into tissues. Finally, UCNPs exhibit neither photoblinking nor photobleaching, and their cytotoxicity is very low. As a result, UCNPs became one of the most promising nanoparticle systems for biological imaging and there are continuing efforts to improve their properties (e.g., increasing luminescent intensity and reducing the particle size) by designing new synthetic strategies. In this study, we demonstrated the benefits of using UCNPs as the probe for real-time imaging and particle tracking in living HeLa cells. Combined with the low cytotoxicity and photostability of UCNPs, NIR excitation enabled uninterrupted long-term imaging of living cells. For the first time, we obtained real-time images of endocytosed UCNPs at the single vesicle level for 6 h continuously at the rate of 20 frames sec^{-1}. The dynamics of particle transport was composed of multiple phases within a single trajectory including the active transport by motor proteins such as dyneins and kinesins.

1004-Pos   Board B790
Photoswitchable Biocompatible Polymer Dots Doped with Diarylethene

Yasuko Osakada[1,2], Lindsey Hanson[1], Bianxiao Cui[1].
[1]Stanford University, Stanford, CA, USA, [2]PRESTO JST, Saitama, Japan.
Molecular photoswitches can be employed for the study of protein trafficking in living cells and applications in optical memories. Especially, to switch fluorescence, fluorescence quenching mechanism via energy or electron transfer is one of the most fundamental pathways to realize the system of photoswitching. In order to achieve fluorescence photoswitching, photochromic compounds such as diarylethene have been used to toggle fluorescence on and off. For example, photochromic diaryethene induces absorption changes upon light irradiations via cyclization reaction, which would trigger the fluorescence toggling. On the other hand, polymer dots (P-dots) is one of the promising fluorescent probes for the biological applications. We assumed that doping diarylethene into P-dots would realize fabrication of photoswhitchable P-dots via energy transfer mechanism between fluorescent polymer and diarylethene. In this study, we synthesized photoswitchable P-dots doped with diarylethene to toggle the fluorescence back and forth via energy transfer mechanism. We also tried to apply synthesized photoswitchable P-dots toward biological imaging. First, we examined the photoswitching properties with absorption and fluorescence measurements. Fluorescence of P-dots was dramatically quenched upon photoirradiation with UV light and recov-

ered after visible light irradiation. Those photoswitching processes were reversible and could go through at least 5 cycles. We are now applying photoswitchable P-dots synthesized as mentioned above to biological imaging. Details will be discussed at the meeting.

1005-Pos   Board B791
Back-Scattered Detection Provides Viable Signals in Many Conditions

Frederick B. Shipley, Ashley R. Carter.
Amherst College, Amherst, MA, USA.
Precision position sensing is required for many microscopy techniques. One promising method, back-scattered detection (BSD), provides position sensing at the level of several picometers, and is compatible with platforms that have restricted optical access (e.g. magnetic tweezers, atomic force microscopy, and microfluidics). However, widespread adoption of BSD may be limited by recent theoretical modeling that predicts diminished signals under certain conditions. In BSD the position of a micron-sized bead is measured by back-scattering a focused laser off the bead and imaging the resulting interference pattern onto a detector. Theoretical modeling of the detector response assumes the bead acts as a Mie-Debye scatterer and creates a first order interference pattern in the back-focal-plane of the collection lens. According to this Mie-Debye scattering model the BSD signal reverses sign many times for bead radii between 100 nm and 2000 nm and that for some radii (e.g. 1000 nm) the BSD response would be vanishingly small, limiting the applicability of BSD. We directly measured the BSD response while varying the experimental conditions, including bead radius, medium refractive index, and numerical aperture of the objective. Contrary to the proposed theory, we find that the signal increases with bead radius. Furthermore, the signal sign does not fluctuate, as predicted, over the tested parameters of radius, numerical aperture, and medium refractive index. We conclude that BSD provides a viable signal in a plurality of conditions.

1006-Pos   Board B792
Use of Fluorescent Sphingolipid Precursors for Biophysical Studies of Sphingolipids

Raehyun Kim, Kaiyan Lou, Mary Kraft.
University of Illinois at Urbana Champaign, Urbana, IL, USA.
Sphingolipids are one of the major components of cell membranes and also play critical roles in cell signaling. Many studies of disrupted cells have expanded our understanding of sphingolipid metabolism and function. Nonetheless, investigations of dynamic sphingolipid events, such as trafficking, diffusion, and organization in cell membranes, require observation of fluorescent sphingolipid analogs within living cells. However, sphingomyelin or ceramide analogs that contain a fluorophore-labeled N-acyl fatty acid cannot be used to track sphingosine or sphingosine-1-phosphate in cells. Additionally, the catabolism of these fluorescent sphingolipids may also result in fluorophore incorporation into glycerolipid species. Though various fluorescent sphingosine analogs have been developed that permit the study of sphingosine and its metabolites, poor photostability of the fluorophores limits long term data collection. Here, we report the use of fluorescent sphingolipid precursors in which a borondipyrromethene (BODIPY) fluorophore is incorporated into the sphingosine backbone. The enhanced photostability of the BODIPY fluorophore improves the ability to observe dynamic sphingolipid events. The fluorescent sphingosine analogs are incorporated into cells by addition to the cell culture media. To verify metabolic incorporation of the fluorescent sphingosine into cellular sphingolipids, lipid extracts from labeled cells were analyzed by thin layer chromatography and mass spectrometry. We demonstrate that the fluorescent analogs of sphingosine can be used to study dynamic events of sphingolipids, such as transport and trafficking.

1007-Pos   Board B793
Exploiting Fluorescence Lifetime Plasticity in Flim: Target Molecule Localisation in Cells and Tissues

Alexander Boreham[1], Tai-Yang Kim[1], Viola Spahn[2], Christoph Stein[2], Lars Mundhenk[3], Achim D. Gruber[3], Rainer Haag[4], Pia Welker[5], Kai Licha[5], Ulrike Alexiev[1].
[1]Institute for Experimental Physics, Freie Universität, Berlin, Germany, [2]Clinic for Anesthesiology, Charité-Universitätsmedizin, Berlin, Germany, [3]Institute for Animal Pathology, Freie Universität, Berlin, Germany, [4]Institute for Chemistry and Biochemistry, Freie Universität, Berlin, Germany, [5]mivenion GmbH, Berlin, Germany.
The mechanisms of drug-receptor interactions and the controlled delivery of drugs via biodegradable and biocompatible nanoparticulate carriers are active research fields in nanomedicine. Many clinically used drugs target G-protein coupled receptors (GPCRs) due to the fact that signaling via GPCRs is crucial