increasing the stability from hours to days. In addition, hybridization with guanine-rich DNA could be used to reduce the already oxidized NCs back to the red-emitting reduced ones. Single-stranded DNA templates were also designed with a nanocluster formation sequence and a guanine-rich sequence at each end. Similarly, we found that the guanine-rich tail helped stabilize the fluorescence of the red-emitting NC fluorophores, in comparison to single-stranded templates with only cluster formation sequences but no guanine-rich tails. Using this strategy, we have designed a DNA sequence that produces a highly emissive Ag NC fluorophore with an extended shelf life, which should prove useful in a variety of biological applications, including fluorescence imaging and biosensing.

3020-Pos

Spectroscopic Characterization of Depolarization Via FRET in a BFP-GFP BoNT/A Assay

Justin A. Ross[1], Nicholas G. James[1], Marcella A. Gilmore[2], Dudley Williams[2], Lance E. Steward[2], Roger Aoki[2], David M. Jameson[1].
[1]University of Hawaii, Honolulu, HI, USA, [2]Allergan Inc, Irvine, CA, USA.

The assay is based on depolarization due to Förster Resonance Energy Transfer (FRET) between Blue Fluorescent Protein (BFP) and Green Fluorescent Protein (GFP) moieties linked by a peptide containing residues 134-206 of SNAP-25, the protein substrate for BoNT/A's proteolytic activity. Before cleavage of this recombinant substrate, the polarization observed for the GFP emission, excited near the absorption maximum of the BFP, is −0.04 due to depolarization following FRET from BFP to GFP. After substrate cleavage and diffusion of the fluorescent proteins beyond the FRET distance, the polarization increases significantly to ~0.4, due to observation of the emission only from directly excited GFP. This change in fluorescence polarization allows for an assay (termed DARET for Depolarization After Resonance Energy Transfer) that is robust and sensitive. In this report we characterize the spectroscopic parameters of the system before and after substrate cleavage, including excitation and emission spectra, polarization and time-resolved methods. Our results suggest that the donor and acceptor dipoles are at a large angle (72°) with respect to one another but that the BFP and GFP are in direct contact in the intact substrate. Evidence is also provided to demonstrate the direct interaction of BFP and GFP after cleavage. We have used this assay to determine the enzyme kinetic parameters (K_m, K_{cat} and V_{max}) for BoNT/A proteolysis of the assay substrate. Our conclusions bear on the issue of the common choice of 2/3 for κ^2 for FRET studies in general and on fluorescent proteins in particular. This work was supported by Allergan Inc.

3021-Pos

A Microfluidic Cytometer for High-Throughput and Quantitative Single Cell Red Fluorescent Protein Photobleaching

Jennifer L. Lubbeck, Kevin M. Dean, Ralph Jiminez, Amy E. Palmer.
University of Colorado, Boulder, CO, USA.

Fluorescent proteins (FPs), given their tendency to convert to a non-radiative triplet state and/or undergo rapid photobleaching, remain sub-optimal for today's advanced microscopy techniques which require low-copy cellular expression, high laser intensities and prolonged imaging durations. To overcome these experimental limitations and address FP photobleaching, which is particularly problematic in orange/red emitting fluorescent proteins, we have developed an innovative microfluidic platform capable of screening 30 mammalian cells per second based upon FP photobleaching. More specifically, the fluorescence intensity of a single hydrodynamically focused cell (5 mm/s) expressing the FP of interest is probed before and after exposure to an intense 1 ms. photobleaching laser ($\approx$ 300 kW/cm2). The resulting emission intensities are measured with excellent sensitivity and signal to noise, permitting the change in fluorescence intensity between the first and second probe beams to be measured with high accuracy. As a result, this platform has enabled us to make quantitative and high-throughput photobleaching measurements as well as differentiate a diverse mixture of red FPs (mOr2, DsRed, mCherry, TagRFP & TagRFP-T). These results provide promising potential for library-based sorting and, given the improved selection criteria, may permit vastly improved photostability in future generations of FP mutants.

3022-Pos

Applications of Pulsed Interleaved Excitation in Live Cell Experiments

Matthias Hoeller[1], Gregor Heiss[1], Kristina Griessmeier[1], Brian Slaughter[2], Katja Straesser[1], Christian Wahl-Schott[1], Don C. Lamb[1].
[1]University of Munich, Munich, Germany, [2]Stowers Institute for Medical Research, Kansas City, MO, USA.

To make Fluorescence Correlation Spectroscopy (FCS) measurements more viable in living cells, various new methods have been developed. Among these are Raster Image Correlation Spectroscopy (RICS), and Scanning Fluorescence Correlation Spectroscopy (SFCS).

In RICS, a confocal raster scanning image of a sample is evaluated to extract concentration, diffusion, or colocalization information of fluorescently labeled molecules using both the temporal and spatial information.

Another advantageous possibility, especially in small organisms, is to use SFCS. There, the confocal laser spot is rotated through a small area, effectively increasing the focal volume and reducing fluorophore bleaching.

Especially in live cell measurements, where fluorescent proteins are typically used, signal levels are often weak, and spectral crosstalk can be a significant problem. Therefore we combined both RICS and SFCS with Pulsed Interleaved Laser Excitation (PIE), a technique we developed to avoid the introduction of artifacts by spectral crosstalk. The sensitivity of both RICS and SFCS to detect dually labeled molecules could be significantly improved, rendering them much more useful for biologically relevant applications both in live cells and in vitro.

The principles of PIE-RICS and PIE-SFCS will be presented along with applications on calcium channels and protein interactions in yeast cells.

3023-Pos

Interpreting FRET in Complex Geometries

Ben Corry, Evelyne Deplazes, Dylan Jayatilaka.
University of Western Australia, Perth, Australia.

Fluorescence resonance energy transfer (FRET) can be utilized to gain low resolution structural information, making use of the fact that the probability of energy transfer is related to the distances between fluorescent molecules. Although the relationship between the efficiency of energy transfer and the distance between sites is well described for a single pair of fluorophores, the situation is more difficult when more than two fluorophores are present. Using a Monte Carlo calculation scheme, we demonstrate how resonance energy transfer experiments can be interpreted when multiple fluorophores are present in complex geometries. We demonstrate the versatility of the approach by calculating the efficiency of energy transfer for individual fluorophores randomly distributed in two and three dimensions, as well as when attached to multimeric proteins. In addition the approach can yield information about the clustering of proteins and their oligomerization state, molecular concentrations and donor:-acceptor ratios.

3024-Pos

Studying Fluorescent Proteins in Living Cells: An Application for Segmented Fluorescence Correlation Spectroscopy

Evan T. Spiegel, P. Lee, L. Toth, W.R. Zipfel.
Cornell University, Ithaca, NY, USA.

Fluorescence intensity fluctuations can provide insight into a multitude of molecular properties from localized concentration and transport mechanisms to characteristic rate constants of reactions. These measurements are possible because the movement of particles in solution gives rise to fluctuations in intensity as the fluorescent species traverse the focal volume. Fluorescence correlation spectroscopy (FCS) is a specialized technique for studying these fluctuations and provides high spatial resolution analysis of molecules at low concentrations.

In homogeneous or even quasi-homogeneous samples, the molecular motions that give rise to these fluctuations are directly related to the molecule of interest. However in more complex environments such as the cell cytosol, there can be an abundance of autofluorescent material, or environment-induced aggregation of the labeled proteins of interest. These sparse, but larger fluorescent species distort the correlation curve by emitting large bursts of photons as they move through the observation region. To overcome this limitation we have developed segmented-FCS (sFCS). In sFCS, the raw data stream is screened for bursts of photons arising from large species allowing the uncontaminated data segments to be isolated. These small segments of the photon stream are then correlated, averaged and analyzed to provide insight into the fluorescent species of interest.

We conducted measurements in RBL-2H3 cells stably transfected with GFP using lab built hardware and software. The customized setup is an economical and efficient solution for a variety of fluorescence measurements and is well suited for the post-acquisition software-based correlation of data. In addition, simulated data confirms the robustness of the sFCS protocol. Our data demonstrates that sFCS can accurately measure concentration and characteristic diffusion rate in contaminated signals and provides a solution for studying fluorescence fluctuations in cells.

(NIH/NCI R01 CA116583 and NIH/NIBIB P41 RR04224.)

3025-Pos

Irregular Excess Energy Transfer Observed with a Cerulean Donor and Multiple Venus FRET Acceptors

Tuan A. Nguyen[1], Srinagesh V. Koushik[1], Paul S. Blank[2], **Steven S. Vogel**[1].
[1]NIAAA/NIH, Rockville, MD, USA, [2]NICHD/NIH, Bethesda, MD, USA.