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

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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

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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

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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

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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

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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.

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3025-Pos

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

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Förster resonance energy transfer (FRET) is a mechanism where energy is transferred from an excited donor fluorophore to adjacent chromophores via non-radiative dipole-dipole interactions. FRET theory primarily considers the interactions of a single donor-acceptor pair. Unfortunately, it is rarely known if only a single acceptor is present in a molecular complex. Thus, the use of FRET as a tool for measuring protein-protein interactions inside living cells requires an understanding of how FRET changes with multiple acceptors. When multiple FRET acceptors are present it is assumed that a quantum of energy is either released from the donor, or transferred to only one of the acceptors present. The rate of energy transfer between the donor and each specific acceptor (k_{DA}) can be measured in the absence of other acceptors, and these individual transfer rates can be used to predict the ensemble FRET efficiency. The generality of this approach was tested by measuring the ensemble FRET efficiency in two constructs, each containing a single fluorescent-protein donor (Cerulean) and either two or three acceptors (Venus). FRET transfer rates between individual donor-acceptor pairs were measured by systematically introducing point mutations to eliminate the chromophores of the other acceptors. We find that the amount of FRET with multiple acceptors is significantly greater than predicted by the sum of the individual transfer rates. We conclude that either an additional energy transfer pathway exists when multiple acceptors are present, or that a theoretical assumption that the prediction calculation is based on is incorrect. These possibilities will be discussed.

3026-Pos

Fluorescence Fluctuation Spectroscopy in the Presence of Hydrodynamic Flow to Determine Protein Stoichiometry at Ultra Low Concentrations

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Fluorescence fluctuation spectroscopy (FFS) provides information about transport parameters, concentration, and interactions of fluorescently labeled molecules. One important FFS parameter is brightness which provides information about the stoichiometry of protein complexes. Here we consider the application of FFS to large protein complexes, such as viruses. FFS measurements of such systems typically require very long data acquisition times due to the low concentration and slow diffusion of the large particles. In order to overcome this drawback we apply hydrodynamic flow, which results in an increased flux of particles passing through the optical observation volume. This technique significantly reduces the data acquisition time of brightness experiments, while extending brightness analysis to femtomolar concentrations. The technique was developed using a test system of fluorescently labeled microspheres flowing through microfluidic channels to investigate the effect flow speed, particle size and brightness on FFS parameters. Finally this technique was extended to determine the copy number of fluorescently labeled Gag protein in viral-like particles present in cell medium. This work is supported by NIH grant R01GM064589.

3027-Pos

Characterization of Conjugated Protein by Molecular Brightness and Mass Spectrometry

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We have previously demonstrated characterization of antigen-antibody interactions with brightness analysis in fluctuation spectroscopy. In these experiments, the antigen studied was labeled with only a single fluorophore. In practice, it may not be straightforward to produce antigen with only a single fluorophore and random conjugation with a dye becomes the simplest approach. To use conjugated protein for single molecule studies, the distribution of conjugate must be understood. Here we introduce a method to evaluate conjugation of proteins using mass spectrometry. We define a mass spread function which describes the distribution of conjugate on a given protein. We show that convolution of this mass spread function with a protein's measured mass spectrum predicts the mass spectrum of conjugated protein. Application is shown using a highly glycosylated antibody with a low amount of incorporated conjugate. For measurement in solution, we use time integrated fluorescence cumulant analysis to characterize conjugated protein in terms of the molecular brightness. We then use brightness analysis to measure concentrations of free and bound conjugated protein in the presence of antibody.

3028-Pos

Combined Optical Tweezers and Fluorescence Microscopy for Single Molecule Experiments

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Optical traps (OT) and single molecule fluorescence (SMF) find use in single molecule biology [1,2]. OT allows manipulation and force spectroscopy of biological macromolecules. Unfortunately both the conformational state of the molecule and force localization on the molecular complex are unresolved in space. SMF characterizes the position of the fluorophore label, yielding the change in shape and conformational state of the labeled molecule. Correlating the force response and label tracking data, the relationship between changes in conformational state and related force are resolved [3]. A combined OT and SMF instrument (OT-SF) allow studying the operational principle of biological molecular motors essential to life.

We implemented SMF imaging into an existing optical tweezer instrument [4]. A diode pumped solid state laser excites fluorescence (Coherent Sapphire 50 mW, 488 nm). Video capture is realized with an intensified CCD camera (Qimaging QICAM Fast). A fluorescence emission filter (Chroma HQ535/30m) maximizes the SNR of fluorescence detection, by maximizing the optical density at the wavelengths corresponding to the trapping and detection lasers. A microscope TIRF objective (Nikon CFI Plan Apo 100X TIRF) facilitates localized excitation in the sample chamber, which reduces the background signal.

Proof-of-principle concurrent SMF imaging and OT micromanipulation of SYBR Gold stained DNA constructs is presented. The proof is presented in the form of a nanoscale video with fluorophore position and forces displayed in real-time. The DNA is bound to optically trapped dielectric beads in a dumbbell configuration.

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3029-Pos

Hyperspectral Line Scanning Microscopy for High-Speed Multicolor Quantum Dot Tracking

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One of the fundamental goals in observing protein-protein interactions on the cell membrane is in achieving nanometer scale spatial resolution along with temporal resolution sufficient to study live cell behavior. Traditional fluorescence microscopy methods have been unsuccessful in studying these interactions due to the diffraction limit with visible light. Single particle tracking techniques using quantum dots have provided single particle localizations to well below the diffraction limit, however, clustering of multiple particles limits the unique identification and thus tracking of individual particles throughout the (possibly dynamic) clustering process. This problem can be solved by tracking multiple quantum dot colors using a high-speed hyperspectral microscope which provides the necessary spatial, spectral, and temporal performance.

We describe a line scanning hyperspectral microscope that uses a prism spectrometer and a fast EMCCD camera to achieve 30 frames per second with 128 spectral channels. We present the optical setup, instrument control and display software and preliminary studies of multi-color quantum dot single particle tracking.

3030-Pos

A Time Resolved Fluorescence Spectrometer with Sub-Millisecond Data Acquisition Time

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We have developed a high-throughput time-resolved fluorescence spectrometer capable of recording a high-resolution time-domain (**sub-nanosecond**) fluorescence decay, with high S/N every 100 μs. Coupled with a conventional stopped-flow rapid mixer, this technology has allowed us to measure changes in time-resolved fluorescence decays occurring during the course of a millisecond-resolved biochemical transient experiment. Most instruments used in fluorescence-based kinetic studies are limited to detecting a single fluorescence intensity signal on the millisecond time scale. While this type of fluorescence intensity-based measurement is informative, it provides scant information compared to a full sub-nanosecond resolved fluorescence decay-which is exquisitely sensitive to the structure, dynamics, and interactions