performing Förster resonance energy transfer (FRET) measurements. Unlike fluorescence intensity which has been traditionally used in FRET experiments, fluorescence lifetimes do not depend on fluorophore concentration, detection efficiency, illumination intensity and uniformity. Therefore, lifetime FRET overcomes the artefacts arising from intensity measurements and provides improved precision in investigating the protein unfolding procedure. Herein, we developed a novel technique for performing lifetime FRET using a maximum likelihood estimator (MLE) adapted from single molecule studies. We demonstrate the feasibility of our detection technique by monitoring the unfolding procedure of the membrane protein Bacteriorhodopsin (bR) labelled with the FRET pair Alexa Fluor 488 (donor) and Alexa Fluor 647 (acceptor). A home-built laser scanning confocal microscope and two avalanche photodiode detectors (APDs) are used for detection with high sensitivity and the fluorescence decays are collected using time correlated single photon counting (TCSPC). When determining a fluorescence lifetime with less than 2000 photons, the conventionally used least squares approach is not appropriate. Therefore, a MLE previously developed in our lab defined by multinomial statistics is used to accurately extract molecular fluorescence lifetimes from as little as 10 photons. These are then used to calculate FRET efficiencies and hence the conformational state of bR.

2999-Pos

Exploiting the Rise Time of Acceptor Fluorescence by FRET-FLIM in Living Cells

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Previously (Borst et al. (2008) Biophys. J. 95) a time-resolved fluorescence methodology has been described for quantitative determination of Förster resonance energy transfer (FRET) between donor-acceptor pairs in biological macromolecules by analyzing the time resolved rise of acceptor fluorescence upon donor excitation. The main advantage is that only those molecules are monitored involved in the energy-transfer process. This contrasts with the more conventional method that measures time-resolved fluorescence of donor molecules and thereby probing a mixture of FRET-active and FRET-inactive populations. We have extended the determination of rise times of acceptor fluorescence to measure FRET in living cells with fluorescence lifetime imaging microscopy (FLIM). Parameters describing the rise of acceptor fluorescence and the decay of donor fluorescence can be determined via simultaneous global analysis of multiple FLIM images thereby increasing the accuracy of the recovered parameters. In the present study, plant protoplasts were transfected with a visible-fluorescent-protein fusion composed of a 6-amino-acid peptide flanked by enhanced GFP (eGFP) and mCherry for illustration of the new data analysis method. It is demonstrated that the distances estimated with the present method are substantially smaller (and more realistic) than those estimated from average donor fluorescence lifetimes. The latter over-estimation is due to a fraction of non-transferring donor molecules, which makes the average fluorescence lifetime of the donor longer. In addition, combining fit results of fluorescence kinetics at different detection wavelengths allows correcting for the contribution of molecules that are not able to transfer their excitation energy. The high fraction of non-transferring eGFP in the construct is explained by taking the presence of non-matured mCherry into account. Software to reproduce the presented results is provided in an open-source and freely available package called "TIMP" for "The R project for Statistical Computing".

3000-Pos

Macrophages Create a Lysosomal Synapse to Digest Aggregated Lipoproteins

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Atherosclerosis is the underlying cause of the majority of heart attacks and strokes. Lipoprotein accumulation and degradation by monocyte-derived macrophages is a central event in the pathogenesis of atherosclerosis. Data show that the vast majority of low density lipoprotein (LDL) in atherosclerotic plaques is aggregated and avidly bound to the subendothelial matrix.

We employ various microscopy and biochemical techniques in both cell culture and animal models to investigate the mechanism of uptake of aggregated LDL (agLDL) by macrophages. We show that macrophages create an extracellular, acidic compartment where the cells contact the agLDL, and lysosomal contents are delivered to these compartments, thereby forming an extracellular hydrolytic compartment - a lysosomal synapse. Active acidification of these specialized compartments and aggregate catabolism were observed by fluorescence ratiometric time-lapse imaging of an *in vitro* cell culture model. Biochemical assays, employing radiolabeled agLDL demonstrated an increase in free cholesterol in aggregates contained in the lysosomal synapse. This cholesterol can be delivered to the cell, initiating the process of macrophage cholesterol loading and ultimately causing progression of the atherosclerotic plaque.

Although *in vitro* systems provide insight into macrophage uptake of lipoproteins, ultimately we must strive to understand the function and behavior of macrophages in intact animals. Relatively little is known of the fate of monocytes once they migrate into atherosclerotic lesions. To this end, we employ intravital multiphoton microscopy in mouse models of atherosclerosis to allow observation of macrophages within the artery wall.

Our studies elucidate the mechanism of a novel pathway for catabolism of agLDL by macrophages. Better understanding of the mechanisms by which macrophages interact with lipoproteins in the subendothelium may lead to new approaches to inhibit lipid accumulation in macrophages and thus, may be of therapeutic value in preventing atherosclerosis.

3001-Pos

Broad-Beam Fluctuation Spectroscopy for Non-Flow Cytometry and Clinical Diagnostics

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Fluorescence fluctuation spectroscopy (FFS) and related techniques such as FCS, PCH and FIDA have been valuable tools for biophysical research, but have not found widespread clinical application. Many clinical blood diagnostics measure the concentration of particular cell types in the blood. These tests are typically performed using fluorescent antibodies and flow cytometry. We present a novel scanning FFS system, which we term Broad-beam Scanning Fluctuation Spectroscopy (BSFS), with application to cytometry. BSFS uses a much larger (~1 nL) observation volume than conventional FCS, so that the fluctuations measured result from cells, rather than individual molecules. This technique is well suited to the measurement of cell concentration, as the correlation analysis also yields a measurement of the sample volume, allowing an absolute concentration to be determined. BSFS is a viable alternative for a variety of cell-based clinical diagnostics, while lacking the optical and fluidic complexity of a flow system.

3002-Pos

Mobility Analysis in Living Yeast using 4Pi CFM

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Continuous fluorescence microphotolysis (CFM) is a powerful tool to analyze molecular mobilities and association reactions in single living cells but has mostly been restricted to diffraction limited focal volumes. Recently, we introduced the theoretical and experimental framework to combine CFM with super-resolution microscopy by utilizing a 4Pi point spread function in mobility analysis.

Here we show that this methodology can be readily applied to living yeast cells under physiological buffer conditions using water-immersion lenses. Yeast cells are relatively small with a typical diameter of 5 μm and the intracellular individual compartmentalization with a large vacuole and the nucleus results in small volume elements with unhindered diffusion. Therefore diffusion measurements with an engineered point spread function should be beneficial to recover the diffusion coefficient as this approach is less susceptible to improper positioning of the laser.

Using 4Pi CFM we were able to clearly recover the diffusion coefficient of GFP in the cytoplasm and the nucleus of living yeast cells. Additionally, the mobility of GFP-tagged proteins involved in nucleo-cytoplasmatic transport was analyzed. While the diffusion coefficient of a GFP-tagged cargo was determined to be in a range expected for a molecule of this respective size, we found evidence that the diffusion coefficient of a GFP-tagged transport receptor was reduced compared to the expected value for purely free diffusion. This might indicate that the molecule is subject to a certain degree of unspecific binding in the cytoplasm of yeast.

3003-Pos

Scanning Laser Image Correlation (SLIC) Measurements in Zebra Fish Larvae

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