the ONM to the INM in real time. This work has been supported by grants from the National Institutes of Health (R01 GM064589, R01 GM098550, RO1 GM124279, R01 GM129374).

886-Pos

Visualizing Dynamic Processes with rapidFLIM^{HiRes}: Ultra Fast Flim with Outstanding 10 PS Time Resolution

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Fluorescence Lifetime Imaging (FLIM) has become an essential tool in Life Sciences over the last decade. However, up to now, users had to choose between high timing precision or fast data acquisition when using Time-Correlated Single Photon Counting (TCSPC) electronics. This was a drawback when investigating fast processes in cells or tissues such as protein interactions, FRET dynamics, or chemical reactions.

We report here on an approach named rapidFLIM^{HiRes}, that allows several FLIM images per second to be acquired with an outstanding temporal resolution of 10 ps. The approach combines the latest advances in fast scanning: Hybrid photomultiplier detectors which are capable of handling very high count rates and TCSPC modules with ultra short dead times and time bin widths as small as 10 ps. Potential decay curve distortions caused by particularly high count rates or detector pulse pile-up are reduced by a suitable correction algorithm.

With rapidFLIM^{HiRes} excellent photon statistics can be achieved in significantly shorter time spans than usually known for FLIM, allowing for the observation of fast processes with the well-known high optical and temporal resolution achievable in confocal microscopy. Depending on the image size, with the presented approach FLIM at video rate is achievable supporting quantitative data analysis even at count rates exceeding 50 Mcps. The capabilities of FLIM^{HiRes} will be highlighted by quantitatively analyzing FRET data obtained from fluorescent proteins in cells.

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Spiral Phase Plate for Tracking 3D Motion in Cells

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Our goal is to map chromatin dynamics in live cell nuclei by tracking 3D lattices of photoactivated chromatin microdomains. We take a multifocal imaging approach to deduce 3D microdomain motions from 2D camera images. Here we report initial results to implement a novel spiral phase plate (SPP) to achieve the 3D-to-2D mapping. The SPP is a diffractive optical element (DOE) designed to create a rotating point spread function (rPSF) in our optical microscope. The SPP consists of a series of helical ramps that provide orbital angular momentum to the emitted fluorescent light. This translates into a pattern of rPSFs in 2D (on a camera chip) that reveals, through their individual centers and angles of rotation, the 3D locations of the chromatin microdomains. We will report on the characterization of the SPP and our initial attempts at tracking chromatin microdomains in 3D. We will compare our 3D tracking results to previous measurements of 2D diffusion in the same U2OS cell line. We will also report on our use of supervised machine learning to accurately identify the source plane along the optical axis of the microscope of each of the observed fluorescent nuclear microdomains (z-position). This technique, combining a SPP and deep learning, should be applicable to any sample consisting of fluorescent objects transversely separated by several microns, such as fluorescently labeled chromatin microdomains in our project.

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The Reliability of Fluorescence Z-Scan Analysis in the Complex Environment of the Living Cell

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Fluorescence z-scan analysis aims to fit the intensity traces recorded while moving a two-photon excitation volume vertically through a cell in order to identify the vertical concentration profile of a fluorescent species. Z-scan analysis has proved able to quantify delicate PM-protein binding interactions as well as sub-resolution partitioning of proteins due to the actin cortex. Despite recent progress, questions remain regarding the reliability and applicability of z-scan analysis in the complex environment of the living cell. In high precision z-scan applications, obtaining a good quality of fit is critical to ensuring that experimental results remain uncontaminated by fluorescent features outside the scope of the z-scan modeling. However, a statistical method to robustly assess the quality of fit in z-scan analysis has been lacking. To address these issues, we provide control data validating core aspects of the z-scan method at high precision and demonstrate the potential for error when applying the method without rigorous quality of fit controls. We propose a conceptual framework for estimating the amplitude of errors in z-scan analysis due to fluorescent features that may not be included in the z-scan fit model. We apply this framework to analyze the potential for microvilli structures, abundant in some cell lines, to perturb z-scan measurements and we outline data quality controls that contain the potential for error. This work provides a foundation supporting the use of simple stratified layers to model concentration profiles within the living cell together with checks that identify when such simplified models may be inapplicable for a given level of precision. This work has been supported by grants from the National Institutes of Health (R01 GM064589, R01 GM098550, RO1 GM124279).

Posters: Single-Molecule Spectroscopy

889-Pos

Single-Molecule Fluorescence-Based Measurements of Conformational Dynamics of Calcium-Binding Protein Recoverin

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Recoverin is a calcium-binding protein expressed primarily in the photoreceptor cells of the vertebrate retina, where it participates in the regulation of phototransduction and adaptation of the visual system to background light. Binding of calcium ions to recoverin induces large conformational changes, including the release of its post-translationally attached myristoyl group from a hydrophobic cavity of the protein into the solution. Previously, it was shown that the fluorophore Alexa647 site-specifically bound to a unique cysteine-39 of recoverin can be employed as a reporter of its Ca^{2+}-dependent conformational transitions since these transitions affect fluorescence lifetime, and spectra of the dye. Here, we addressed a question, whether the Ca^{2+}-dependent conformers of recoverin, previously resolved by NMR, can be recorded using single-molecule fluorescence microscopy. To this end, the samples of recombinant bovine recoverin site-specifically labeled at cysteine-39 with either Alexa647 or sulfo-Cy5 maleimide-functionalized fluorophores were prepared. Both forms of the protein exhibited Ca^{2+}-induced conformational changes as revealed by steady-state spectrometry of intrinsic tryptophans fluorescence. Interestingly, two chemically similar cyanine dyes (Alexa647 and sulfo-Cy5) prodruced quite different effects on recoverin state and exhibited different photophysical responses to changes in its conformation. We performed single-molecule experiments with recoverin fluorescently labeled with Alexa647. Using a confocal setup with multiparameter fluorescence detection (MFD) we measured fluorescence lifetimes and anisotropies of bursts from individual recoverin molecules. Observation of individual molecules in solution is limited by their diffusion time (typically, 1-10 ms) - to track slower conformational changes we specifically immobilized recoverin using a biotin-streptavidin tether. Using a TIRF microscope we measured intensity traces from individual immobilized molecules. Our results show that conformational dynamics of recoverin can be measured at the single-molecule level using the protein site-specifically labeled at cysteine-39 with Alexa647. This work is supported by RFBR (№20-34-70034).