state to a fluorescent state. Only a small fraction of the molecules are activated at any given time; giving a very low probability of nearby molecules being simultaneously activated and forming overlapping blurs in the image. The fluorescent molecules are imaged individually, their positions are determined from the images, and then a new set of molecules is activated to the fluorescent state. However, there is a tradeoff between image quality (improved by minimizing the activation probability per molecule, for fewer overlapping blurs) and speed (improved by increasing the activation probability per molecule, to reduce the risk of image cycles with zero activated molecules). One method of dealing with this tradeoff is to increase the activation probability and use algorithms (called rejection algorithms) that identify and remove spots formed by overlapping blurs from more than 1 molecule.

We performed a theoretical analysis to relate rejection algorithm performance with achievable resolution and image acquisition speed. We predict the existence of a minimum acquisition time independent of algorithm performance, and an algorithm-dependent maximum error rate. We have characterized the performance of commonly-used procedures for identifying multi-molecule spots via their shape (including linear and non-linear curve fitting), and show that procedures of widely varying complexity and speed have comparable performance, pointing to ways of reducing acquisition and post-processing time with optimized rejection algorithms. Additionally, we analyzed errors when molecules produce overlapping blurs and are then bleached. With proper control of activation probability and the photobleaching rate, bleaching can actually be used to enable faster acquisition of an image with subwavelength resolution, with implications for the design of photoswitchable fluorescent proteins.

937-Pos

Imaging Total Internal Reflection Fluorescence Cross-Correlation Spectroscopy (ITIR-FCCS)

Jagadish Sankaran[1], Manoj Manna[2], Lin Guo[2], Rachel Kraut[3], Thorsten Wohland[1].
[1]Singapore-MIT alliance and Department of Chemistry, National University of Singapore, Singapore, Singapore, [2]Department of Chemistry, National University of Singapore, Singapore, Singapore, [3]School of Biological Sciences, Nanyang Technological University, Singapore, Singapore.

General imaging approaches, in which contrast is not given by time averaged intensities but by other fluorescence parameters, for instance lifetime, anisotropy or parameters of correlation functions, promise to give new insights to biologists. We have recently shown that Fluorescence Correlation Spectroscopy (FCS) can be performed using EMCCD cameras if used with an objective type total internal reflection illumination scheme. This so called Imaging Total Internal Reflection-Fluorescence Correlation Spectroscopy (ITIR-FCS), allows the measurement of autocorrelation functions (ACF) on thousands of pixels independently on 2D surfaces. In this work we extend this technique to ITIR-FCCS (Fluorescence Cross-correlation Spectroscopy) to perform spatial cross-correlation for the measurement of general translational processes. A generalized expression was derived for auto- and cross-correlations of arbitrarily shaped areas on a CCD for diffusion and flow processes. ITIR-FCCS was able to precisely and accurately determine flow velocities and diffusion coefficients of model systems.

To address the translational processes and the organization of cell membranes we calculated the differences between spatial forward and backward cross-correlations to yield so called ΔCCF images. The ΔCCF imaging approach was successfully demonstrated on GUVs and mixed lipid bilayers by demarcating phase boundaries. It was then used to track the changes in heterogeneity of two cell membrane markers, a liquid-ordered phase marker (sphingolipid binding domain (SBD) derived from the amyloid peptide Aβ) and a liquid-disordered phase marker (DiI) on live neuroblastoma cells under conditions of cholesterol depletion and cytoskeletal disruption. Our findings from auto- and cross-correlation and ΔCCF analysis indicate that SBD is influenced by the cholesterol content and to a larger extent by the integrity of the cytoskeleton. DiI, by contrast, shows little dependence on both.

938-Pos

Optical Nanoscopy Far-Field Approaches to Cellular and Molecular Biophysics

Alberto Diaspro[1], Paolo Bianchini[1], Francesca Cella[2], Emiliano Ronzitti[2,3], Silvia Galiani[2], Mattia Pesce[1], Zeno Lavagnino[2], Gaser Abdelrasoul[1].
[1]IIT - Italian Institute of Technology, Genoa, Italy, [2]IFOM MICROSCOBIO and Dept.Physics-University of Genoa, Genoa, Italy, [3]SEMM-IFOM-IEO University of Milano, Milano, Italy.

Fluorescence optical far-field microscopy fostered the design and realization of crucial experiments in cellular and molecular biophysics, although the limited spatial resolution dictated by diffraction. Recently, an emerging family of fluorescence microscopy approaches exploiting the photo physical properties and the switching abilities of fluorescent markers allowed to achieve the surpassing of the diffraction barrier down to 10 nm resolution scale. Super-resolution microscopy and optical nanoscopy are the modern terms related to optical far-field methods opening a new window for the understanding of molecular interactions within the biological cell (A. Diaspro (ed.) (2009) ''Nanoscopy and Multidimensional Optical Fluorescence Microscopy '', Chapman and Hall). Within this framework, focusing on the saturated depletion of the markers' fluorescent state by stimulated emission we have pointed our attention to different modalities for realizing STED (stimulated emission depletion) approach. In particular we are interested in the excitation modalities (including phase modeling, intensity control and scanning speed) and in the possible photo-bleaching/toxical effects as function of the light intensity levels needed. To this end we are working on the ''classical'' solution using ps laser pulses both using white light laser generation and multi-photon based schemes as compared to the continuous wave (CW) excitation and depletion achieved by means of CW laser sources. In parallel we are also approaching optical super-resolution using the FPALM (fluorescence photoactivatable localization microscopy) scheme coupled to two different ways for switching on the fluorescent proteins involved. The former being classical, in order to have a comparison with the STED approach in terms of possible photo-bleaching and photo-toxical effects, and the latter based on the utilization of the single plane illumination microscopy (SPIM) concept to extend far-field optical nanoscopy methods to large samples.

939-Pos

Recognition of Protein Binding Events by Polarity-Sensitive Probes

Ranieri Bizzarri[1,2], Giovanni Signore[3], Riccardo Nifosi[2], Lorenzo Albertazzi[1,3], Barbara Storti[1,3].
[1]NEST, Scuola Normale Superiore, Pisa, Italy, [2]NEST, CNR-INFM, Pisa, Italy, [3]NEST @ Italian Institute of Technology, Pisa, Italy.

Polarity-dependent fluorescent probes are recently attracting interest for high-resolution cell imaging. The fluorescence enhancement of the solvatochromic dye, ideally located in a domain where polarity changes occur upon binding, allows for a fine detection of molecular recognition events even between non overexpressed proteins.1 We developed a toolbox of new solvatochromic coumarin derivatives, characterized by a donor-(coumarin core)-acceptor structure, tailored to in vivo imaging applications.

After a preliminary screening by computational methods, we adopted a synthetic procedure tuneable on the substitution patterns to achieve. Our probes possess excellent fluorescence quantum yields (up to 0.95), high molar extinction coefficients (up to 46,000 M-1cm-1), and large Stokes shifts. Furthermore, they display strong solvatochromism, being almost non emissive in water and very fluorescent in less polar media (up to 780-fold enhancement in brightness). When tested on cultured cells, the developed coumarins resulted not harmful and their photophysical properties were unchanged compared to free solution. Due to both their strong solvatochromic properties, and their lipophilic character, the coumarin did fluoresce only in the most lipophilic environments of the cell. In particular, colocalization experiments with standard markers evidenced staining in ER, membranes and lysosomes, depending on the chemical structure of the solvatochromic probe.

Finally, one compound (3-benzothiazenyl-4-ciano-6,7-dimethoxy coumarin) showed monoexponential decay of fluorescence with a lifetime which is linearly dependent on solvent polarity. This feature promotes its use as ratiometric indicator of cell polarity at nanoscale level. The prepared compounds are remarkable tools to investigate subtle biochemical processes in the cell environment after appropriate conjugation to biomolecules, and at the same time constitute the basis for further engineering of a new generation of biosensors.

1) Nalbant, P.; Hodgson, L.; Kraynov, V.; Toutchkine, A.; Hahn, K. M. Science 2004, 305, 1615-1619.

940-Pos

Superresolution Microscopy with Conventional Organic Fluorophores

Mike Heilemann[1], Sebastian van de Linde[1], Ulrike Endesfelder[1], Anindita Mukherjee[1], Steve Wolter[1], Markus Sauer[2].
[1]Bielefeld University, Bielefeld, Germany, [2]Julius-Maximilians-University Wuerzburg, Wuerzburg, Germany.

Fluorescence microscopy is a sensitive and non-invasive tool to study biomolecular structure and interactions. However, a diffraction limit resulting from the wave nature of light limits resolution to ~200 nm in lateral and ~700 nm in axial direction.

Out of a large set of methods that bypass the resolution limit and open the door for diffraction resolution microscopy, many of them rely on the use of photoactivatable or photoswitchable molecules, combined with precise single-molecule localization and image reconstruction. This concept has recently been extended to a large set of commercially available fluorophores (1-3). Key issues that need to be addressed are (i) controllable switching rates, (ii) live-cell