Optical Microscopy and Super Resolution Imaging III

3040-Pos Board B732
Exploiting Binding Kinetics of Fluorogen Activating Peptides to Enhance Photostability: Applications to Live Cell Single Molecule Imaging
Saumya Saurabh1, Victor R. Mann1, Lauren E. Beck1, Ming Zhang1, Andrea Costello1, Marcel P. Bruchez2.
1Chemistry, Carnegie Mellon University, Pittsburgh, PA, USA, 2Physics, Carnegie Mellon University, Pittsburgh, PA, USA.

Inefficiency of fluorescent labels imposes key limitations in fluorescence microscopy. Photobleaching, non-specific signal from multiple fluorophores, and lack of multiplexing and orthogonal labels impose major challenges in studying complex cellular systems, both at ensemble and single molecule levels. We have addressed these problems by exploiting the binding kinetics of fluorogenic activating peptides that on binding to a non-fluorescent molecule (a fluorogen) exhibit fluorescence. Novel methods in flow cytometry and rational design of peptides enabled the elucidation of peptides and fluorogen pairs with varying off rates. Complexes with fast off rates (koff=5E-3 s^-1) can exchange a bleached fluorogen molecule with a fresh fluorogen molecule in solution. Since the free fluorogen is dark, we can image for extended time scales in the presence of excess fluorogen. For complexes with slow off rates (koff=5E-5 s^-1) and high affinity (Kd<1E-9M), we can target commercially available Streptavidin QDots to proteins on the cell membrane, cytoplasm and the nucleus. We have imaged these proteins at a single molecule level for long time scales by exploiting the exceptional photostability of QDots. We have shown the use of this technique in particular, to the study of the diffusion dynamics of the β2 adrenergic receptor and a trans-membrane protein on the cell surface simultaneously in real time. We have also developed peptides that non-covalently target cyanine dyes with sub-nanomolar affinities. This solves the problems associated with protein-dye conjugation and enhances the photostability of these dyes up to ten fold. We have demonstrated the use of these peptides in single molecule studies of membrane proteins. Thus by exploiting kinetics, we have developed a genetically encoded toolbox for targeting fluorogens, organic dyes and QDots for the study of proteins on and inside the cell.

3041-Pos Board B733
Quantitative Retardance Imaging using Quadri-Wave Lateral Shearing Interferometry (QWLSI)
Sherezade Aknoun1, Pierre Bon1, Julien Savatier1, Benoit Wattellier1, Serge Monneret1.
1Mosaic team, Institut Fresnel, Marseille, France, 2PHASICS S.A., Palaiseau, France.

We describe the use of quantitative phase imaging to enhance specifically the contrast of ordered components like fibers and membranes inside biological samples. Phase separation is achieved by recording a pair of images for the label-free and long-time duration imaging of semi-transparent biological samples. Recent techniques give access to a quantitative measurement of optical thickness. Changes in the refractive index inside the samples can be the main contrast sources in living cells. It is known that some biological structures inside cells are optically anisotropic and thus scatter light differently depending on the illumination light polarization. This property is widely used in polarized light microscopy to reveal ordered structures without staining or labeling but most techniques are purely qualitative or hard to implement when one wants to obtain quantitative measurements on living specimens. Measurement of phase shifts introduced with different incident polarization angles, would give access to quantitative values of both linear retardance and orientation of optical axes. We propose here to use QWLSI to measure a set of polarization-dependent phase shifts, in order to reveal collagen fibers local structure and some components like actin stress fibers in living cells samples. The high-resolution wavefront sensor is mounted on a non-modified transmission microscope to measure characteristic optical path difference of the sample [1]. The very simple setup is composed by a single rotating polarizer placed in the illumination light path before the sample that leads to record one quantitative phase image for each excitation polarization angle. The set of those images, recorded in few seconds so as to deal with living samples, is numerically computed to obtain what we call “Quantitative Retardance Images” which represent the local retardance value of the sample and its optical axis local orientation.

3042-Pos Board B734
Quantification of Co-localisation; Co-Occurrence, Correlation, Empty Voxels, Regions of Interest and Thresholding
Jeremy Adler1, Ingela Parnym2.
1Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden, 2Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden.

Measuring co-localisation is not straightforward with a plethora of coefficients that encapsulate different definitions. Measurements may also be implemented differently. Not only do measurements differ; interconversion is impossible making comparisons challenging. There is a need to cull coefficients and for clear definitions of what precisely is meant by co-localisation in individual studies. Co-localisation can be considered to have two components; co-occurrence which reports whether the fluorophores are found together and correlation which reports on the similarity in their patterns of intensity. Pairs of molecules can show complete co-occurrence but nonetheless be uncorrelated. Co-occurrence can be measured by area or using the M1 and M2 coefficients and should be compared to random distributions. Correlation analysis should use the Pearson and Spearman coefficients preferably in combination with replicate based noise corrected correlation to eliminate errors arising from noise. Ideally, both co-occurrence and correlation should be assessed. Coefficients not mentioned above do not clearly report either co-occurrence or correlation making them hard to interpret. Quantitation depends upon differentiating between background and signal and we describe how this can be achieved. The local mean threshold can not since it distorts the intensity relationships. Measurements should be made within biologically relevant regions of interest (ROI) and the correlation may differ between whole image, whole cell and organelle ROIs. A contentious point is how to handle pixels in which neither fluorophore is present. We demonstrate why negative correlations calculated between populations of voxels in which the two fluorophores never co-occur are completely meaningless. Another issue is whether cellular distributions only arise from interactions between the two molecules. Distributions could also reflect physicochemical properties, substantial biological inhomogeneity, targeted delivery and retention. In summary, we need a unified terminology and stricter editorial policies for reporting co-localisation.

3043-Pos Board B735
Nanoscale Protein Diffusion by Sted-Based Spatiotemporal Fluorescence Correlation Spectroscopy
Paolo Bianchini1, Francesco Cardarelli1, Mariangela Di Luca1, Alberto Diaspro1, Ranieri Bizzarri2.
1Nanophysics, Istituto Italiano di Tecnologia, Genoa, Italy, 2Center for Nanotechnology Innovation @NEST, Istituto Italiano di Tecnologia, Pisa, Italy, 3NEST, Scuola Normale Superiore and Istituto Nanoscienze - CNR, Genoa, Italy, 4Istituto di Biofisica - CNR, Pisa, Italy.

Fluorescence Correlation Spectroscopy (FCS) represents an established technique to recover single-molecule diffusion and binding properties in cells. Recently, scanning microscopy imaging was applied to add a spatial dimension to the classic, purely temporal, FCS modality: spatiotemporal FCS (stFCS) provides details about the routes that diffusing particles or molecules follow in the specimen.[1] We report on combining spatiotemporal fluorescence correlation spectroscopy (stFCS) and stimulated emission depletion (STED) to monitor intracellular protein diffusion at a spatial resolution below the optical diffraction limit (super-resolution)[2,3]. We validated our method both in vitro and at intracellular level, studying the diffusion of fluorescent nanocapsids and of GFP bound to SV40 Nuclear Localization Signal (NLS), respectively. NLS-GFP represents a well-known model of actively nuclear-imported protein that has been the subject of intense research.[4] We demonstrated how relevant is our approach discovering persistent complexes between nucleo-cytoplasmic transporters and NLS-GFP at distances >500 nm from the nuclear envelope. Such a phenomenon would be otherwise invisible at the best resolution of conventional confocal imaging mode. We should stress that, in principle, the photophysics of the fluorescent reporter in STED conditions is the only limiting the resolution in stFCS diffusional maps.[5] 1. M. A. Digman and E. Gratton, Annu Rev Phys Chem 62, 645-668 (2011). 2. F. Cardarelli, R. Bizzarri, M. Serresi, L. Albertazzi, and F. Beltram, J Biol Chem 284(32), 36638-36646 (2009). 3. A. Diaspro, Nanoscopy and Multidimensional Optical Fluorescence Microscopy, 448 (Chapman and Hall/CRC, Boca Raton, USA, 2010). 4. S. Galiani, B. Harke, G. Vicidomini, G. Lignani, F. Benfenati, A. Diaspro, and P. Bianchini, Opt Express 20(7), 7362-7374 (2012). 5. S. W. Hell, Science 316(5828), 1153-1158 (2007).