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Mapping Diffusion in a Living Cell using the Phasor Approach

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The diffusion of a fluorescent protein within the cell has been measured by using either the fluctuation based techniques (FCS, RICS) or through particle tracking or through FRAP. However none of these methods enable us to measure the diffusion of the fluorescent particle at each pixel of the entire image and create a detailed diffusion map of the cell. Measurement using the conventional single point FCS at every individual pixel results in the long exposure of the cell to the laser and eventual bleaching of the sample. To overcome this limitation we have developed new modes of scanning. In this new method of modified raster scanning, the laser scans each individual line multiple times before moving to the next line. The difference from the RICS approach is in RICS the data is acquired by scanning each frame once and then scanning the image multiple times. The other mode resembles single point FCS at each point of an image, albeit for a very short time. The total time of data acquisition required for these acquisitions are much shorter than the traditional FCS analysis at each pixel. However, at single pixel the acquired intensity time sequence is short; requiring a non-conventional analysis of the correlation function to extract information about the diffusion. The phasor approach, a fit-free method that was originally developed for the analysis of FLIM images was applied to analyze the obtained correlation functions. Analysis using this method results in an estimation of the average diffusion coefficient of the fluorescent species at each pixel of an image, and thus a detail diffusion map of the cell can be created.

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The availability of a system such as the MS2-GFP fusion protein, which directly labels the mRNA, has allowed obtaining an estimation of the RNA polymerase elongation speed in living cells. There is however a large heterogeneity observed in RNA Polymerase II (PolII) elongation rates measured from fluorescence assays.
To shed further light on the source of this heterogeneity we introduce and discuss here a novel method based on the phasor analysis of steady state MS2-mRNA fluorescence trajectories. When applied to the study of PolII kinetics, we demonstrate that this approach allows resolving PolII elongation rates in a range from a few to hundreds of basepairs per seconds.
In order to couple this information to what happens to mRNA molecules once they leave the active transcription site, we combine 3D orbital particle tracking with Pair Correlation Analysis to investigate the diffusive routes taken by mRNA molecules within the nucleoplasm. With this approach we observe that the time an mRNA molecule takes to leave the transcription site is highly variable, ranging from a few to tens of seconds. Work supported in part by Grants NIH P41-GM103540 and NIH P50-GM076516

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Nanoscale Protein Diffusion by STED-Based Pair Correlation Analysis
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We describe for the first time the combination between cross-pair correlation function analysis (pair correlation analysis or pCF) and stimulated emission depletion (STED) to obtain diffusion maps at spatial resolution below the optical diffraction limit (super-resolution). Our approach was tested in systems characterized by high and low signal to noise ratio, i.e. Capsid Like Particles (CLPs) bearing several (>100) active fluorescent proteins and monomeric fluorescent proteins transiently expressed in living Chinese Hamster Ovary cells, respectively. The latter system represents the usual condition encountered in living cell studies on fluorescent protein chimeras. Spatial resolution of STED-pCF was found to be about 110 nm, with a more than twofold improvement over conventional confocal acquisition. We successfully applied our method to highlight how the proximity to nuclear envelope affects the mobility features of proteins actively imported into the nucleus in living cells. Remarkably, STED-pCF unveiled the existence of local barriers to diffusion as well as the presence of a slow component at distances up to 500-700 nm from either sides of nuclear envelope. The mobility of this component is similar to that previously described for transport complexes. Remarkably, all these features were invisible in conventional confocal mode.

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Analysis of Trabecular Bone Architecture using Two Photon Fluorescence Microscopy
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Biomechanical competence of trabecular bone is dependent on the makeup of its architecture. Alterations in the trabecular architecture can lead to fractures in metabolic bone diseases like osteoporosis. Therefore, it is paramount to understand the signaling mechanisms that dictate these changes in bone growth and fracture repair. Two photon fluorescence microscopy revolutionized the imaging of biological specimens utilizing its unique capabilities. The three-dimensional (3D) imaging based on nonlinear excitation of the fluorophores brings multiple advantages for imaging skeletal tissue. However, noise generated by the subsurface signal and auto-fluorescence of the local tissue make imaging of trabecular bone problematic. Imaging of calcified tissue presents a unique challenge to address the aberrations produced through the noise generated. Also a general practice of immunolabeling of the plasticized bone for antigen stability are to be optimized. We demonstrate here for the first time using two-photon fluorescence imaging of trabecular bone and its architecture identifying the structural differences and cell populations lining the trabecular cavity and also the cells embedded in it. Furthermore, we developed a shortened method of immunohistochemistry for plastic embedded bone tissue providing antigen stability for antibody labeling. Two photon fluorescence imaging greatly reduces photo damage and helps image of specimens of uneven planes to submicrometer resolution making this an ideal source for imaging in vivo signaling of trabecular bone. We demonstrate here labelling of multi colored fluorophores measuring Smad and ERK activity in trabecular bone growth in mice that are systemically injected with Bone Morphogenetic Protein 2 (BMP2). We optimized the conditions for in vivo imaging of bone tissue that is calcified and plasticized. We demonstrate here two photon fluorescence microscopy of the trabecular bone can be used for understanding the molecular mechanisms which control bone growth and development in vivo.

1632-Pos Board B583
Non-Linear Microscopy of Mitochondrial Damage and Abnormal Lipid Metabolism in Beta-Amyloid Expressing Yeast
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One of the earliest pathological hallmarks of Alzheimer's disease is the formation of soluble β-amyloid (Aβ) oligomers, also believed to be the primary neurotoxic agents long before the accumulation of amyloid plaques. However, the mechanisms by which the Aβ oligomers cause cell dysfunction and eventually cell death are poorly understood. The yeast *Saccharomyces cerevisiae* has here emerged as a valuable model for systemic studies of the intracellular cytotoxicity of Aβ species, revealing that Aβ transits through the different endocytic compartments and disrupts cell-, mitochondrial-, lysosomal- and ER membranes (for a review of the different aspects of amyloid-membrane interactions) finally activating the mitochondrial apoptotic pathway. In order to form a deeper understanding for the cause and consequences of mitochondrial damage, seemingly one of the central cytotoxic mechanisms, we have done a multi-parametric study on living GFP-Aβ42 expressing yeast using non-linear microscopy. The intracellular distribution of GFP-labelled Aβ42 was correlated with the corresponding distribution and morphology of mitotracker-labelled mitochondria by means of 2-photon fluorescence microscopy. Furthermore, the consequences of the dysfunctional mitochondria and the resulting oxidative stress were visualized by the monitoring of the general NADH levels based on their 2-photon-excited intrinsic fluorescence and the content and distribution/morphology of lipid stores by means of CARS microscopy (probing natural carbon-hydrogen vibrations). We could observe how Aβ-expressing yeast accumulates significant amounts of lipid stores and follow their coalescence to larger store units, which can be recognized as a general stress response, in this case most likely due to oxidative stress.

1633-Pos Board B584
Using Surface Plasmon Resonance to Study Species Transport across Lipid Membranes
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Studying species transport across lipid membranes by membrane transport proteins is important for various biological applications. Although patch-clamp technique is well developed for recording the ion transport across lipid membranes, the technique requires well trained personals for the challenging and delicate operation. In this study, we demonstrated using the surface plasmon resonance (SPR) based platform to detect the concentration change of the target species across the lipid membrane. We created sub-micron sized pore structure on the platform, in which the bottom surface is gold and the top surface is silica, and spanned lipid membranes over the pore. The process created a space inside the pore separated from the outside environment by the free-standing lipid membrane for further studying the species transport across the membrane. The platform geometry allowed us to combine plasmon-waveguide resonance (PWR) to the system to simultaneously monitor the refractive index change in the pore space, which is correlated to the target species concentration, and the refractive index change on the membrane above the top silica surface, which is correlated to the binding events occurring on the membrane surface. We expect to use this platform to monitor how various inhibitors or ligands could influence the transport dynamic of interested membrane transport proteins.

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Applications of High Resolution Surface Plasmon Resonance Imaging to Adherent Cells: Single Mammalian Cells to Bacterial Biofilms
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High resolution surface plasmon resonance imaging (SPRI) allows label-free imaging of subcellular features when performed using a high numerical aperture objective lens with a digital light projector to precisely position incident angle excitation. The SPRI signal is a result of the mass of material within