this study are generally applicable for studying diffusion and exchange of membrane associated fluorophores using FRAP on widely available commercial confocal laser scanning microscopes.

166-Pos Board B45

Dynamic Spatial Distribution of RNA Polymerase in Live E. coli

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Escherichia coli is one of the few model systems that has been extensively studied in biology. Because of their small size, bacterial cells have always been difficult to study with light microscopy. Electron microscopy can give spectacular images of the static structure of fixed bacterial cells, but fluorescence microscopy provides quantitative information about subcellular structure and dynamics in living cells.

One of these quantitative methods is fluorescence recovery after photobleaching (FRAP). FRAP can be used to study dynamic redistribution of fluorescent tracer particles in cells. Of interest to our lab is *E. coli* RNA polymerase, the enzyme responsible for transcribing DNA into RNA. FRAP provides information on the diffusion of polymerase on the ~1 μm length scale. We have measured the fluorescence distribution recovery in live *E. coli* held at 30°C in flowing aerated growth media. Preliminary results indicate that there is a population of fluorescently tagged RNAP that recovers on the time scale of seconds. This leads to a rough estimate of $D_{apparent}$ ~0.2 μm^2-s^{-1}. As a comparison, the mean *in vitro* 1D sliding diffusion constant of T7 RNAP as reported by Kim and Larson in 2007 was 0.12 μm^2-s^{-1}. Somewhat surprisingly, it appears as if RNAP can diffuse as quickly inside the cell with its hop and slide method as it can outside the cell with 1D sliding. Actively transcribing polymerases should appear stationary over this time scale. This allows us to estimate the fraction of actively transcribing RNA polymerase, or otherwise immobile, to be about 0.6.

167-Pos Board B46

Morphogen Gradient Formation Unraveled Using In Vivo Three-dimensional Single Molecule Microscopy

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Positional information is essential for the cell's fate in tissue. In the wing imaginal disk of Drosophila melanogaster positional information is provided by a concentration gradient of the morphogens Decapentaplegic (Dpp) and Wingless. We use a 3D-epifluorescence setup to unravel the spatio-temporal distribution of YFP-labeled Dpp after secretion by specialized producing cells. With our approach we are able to characterize the Dpp distribution in the wing disk in all three dimensions in vivo. Most Dpp is located apically in a layer of ~5 μm. To elucidate how the gradient is maintained individual endosomes containing Dpp are followed. We found that endosomes contain up to 100 Dpp molecules allowing us to follow endosomes for hundreds of frames with high spatio-temporal accuracy in three dimensions.

The Dpp concentration in each endosome was directly determined from the fluorescence intensity. We find a constant Dpp fraction of 60% in endosomes, agreeing with the fixed fraction found in FRAP experiments. Sudden changes in Dpp content of up to 15 Dpp molecules are observed, indicating that Dpp is endocytosed in clusters into vesicles before vesicle fusion with endosomes occurs. Surprisingly multiple preferred Dpp cluster sizes are found. Measuring Dpp in- and outflow results in rates on the order of minutes. Labeling different types of endosomes allows us to calculate Dpp degradation and recycling rates. Endosome mobility plays an important role in maintaining the Dpp gradient. We find that Dpp-containing endosomes close to the Dpp source are transported during 15% of the time. Further away this percentage drops, indicating that transport via endosomes is playing a less important role in maintaining the morphogen gradient. Our study leads to a mechanistic model for gradient formation on the level of the mobility of individual Dpp endosomes and molecules.

168-Pos Board B47

$G\alpha_q$ Binds Two Effectors Independently in Cells: Evidence for Predetermined Pathways

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G proteins transduce signals along diverse pathways, but the factors involved in pathway selection are largely unknown. Here, we have studied the ability of $G\alpha_q$ to select between two effectors, phospholipase Cβ (PLCβ) and phosphoinositide-3-kinase (PI3K). Specifically, we expressed eCFP and eYFP tagged proteins in HEK293 cells and monitor their interactions throughout stimulation using Forster resonance energy transfer (FRET). We find separate and stable pools of $G\alpha_q$-PLCβ and $G\alpha_q$-PI3K complexes existing in both the baal and stimulated states. These separate complexes exist despite the ability of $G\alpha_q$ to simultaneously bind both effectors as determined by *in vitro* measurements using purified proteins. Pre-formed G protein-effector complexes will limit the number of pathways a given signal will take and may simplify predictive models.

169-Pos Board B48

Trafficking Of Glutamatergic And Peptidergic Vesicles In Astrocytes

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In neurodegenerative disorders and in trauma of the central nervous system (CNS) excitotoxic stress is developed due to highly increased extracellular concentrations of neurotransmitters. Astrocytes are, in addition to neurons, sensitive to excitotoxic stress, leading to an increase in the intracellular free calcium concentration ([Ca2+]i). This elicits a discharge of several gliotransmitters from membrane-bound vesicles and probably also affects the pattern of vesicle trafficking in astrocytes. Several aspects of the trafficking of membrane-bound vesicles in astrocytes have been studied, but their recycling is poorly defined. We labeled recycling vesicles containing either the vesicular glutamate transporter 1 (VGLUT1) either vesicles containing atrial natriuretic peptide (ANP). We examined their number, fluorescence intensity and mobility by confocal microscopy. A rise in [Ca2+]i elicited an increase in the number and fluorescence intensity of the puncta. In contrast to non-stimulated cells, where VGLUT1 vesicles cycle slowly between the plasma membrane and the cytoplasm, in stimulated cells many vesicles exhibited higher, directional mobility. The opposite effect of stimulation was measured for ANP-vesicles. In CNS pathologies astrocytes change the expression of many genes, including genes encoding intermediate filament proteins. Since cytoskeleton-severing agents abolished vesicle mobility, this indicates a cytoskeleton dependent vesicle recycling. Our findings importantly contribute to the understanding of how vesicle mobility is regulated.

170-Pos Board B49

Developing Statistical Diagnostic Tools For Discriminating Between Different Diffusive Modes Of Fluorescently Tagged Protein Complexes In Living Cells For Short Duration Trajectories

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Several protein molecular complexes in living cells are known to diffuse in many different modes. These include normal Brownian diffusion, anomalous or sub-diffusion, confined/picketed diffusion and facilitated diffusion. Such a variety of diffusive modes belies the heterogeneity in the cellular environment, both in terms of effective viscosity of the intracellular medium and marked differences in packing densities and spatial organisation of molecular substructures, both in the cell membrane and the cytoplasm. Discriminating diffusive modes is relatively easy for long duration trajectories, however obtaining such trajectories typically requires a relatively cumbersome tag, such as using colloidal gold beads of several 10s of nm is diameter, or functionalised quantum dots again in excess of 10nm in effective diameter, which inevitably affects normal physiological function and diffusion and may lead to misinterpretation of the underlying biology. This has applications in topical questions such as the degree to how freely mixed the membrane is or whether interacting proteins are confined through the membrane micro-structure. A far better approach is the use of smaller fluorescent tags under more physiological conditions, such as genomically-encoded fluorescent protein fusions such as GFP. However, such fluorescent proteins have photophysical properties that typically only allow optical tracking over short time windows for tracked protein complexes. Here we present new statistical approaches which allow discrimination of different diffusive modes on such short duration trajectories.

171-Pos Board B50

Probing the Intracellular Reaction Dynamics of Low Density Lipoprotein Using Single Particle Tracking Fluorescence Microscopy

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Interactions between substrate-containing late endosomes and enzyme-containing lysosomes mediate intracellular reactions that lead to the degradation of the substrate. To monitor the intracellular degradation of low density lipoprotein (LDL) in live cells, individual LDL particles were labeled with approximately 200 lipophilic, fluorescent dye molecules. Due to the close proximity of individual fluorophores, the emission of photons by the fluorophore is quenched. Upon enzymatic degradation, the LDL particle exhibits a 3-fold fluorescence

enhancement. The change in fluorescence intensity of the LDL particle is observed with fluorescence microscopy and analyzed using single particle tracking. Dequenching events are observed as a single-step increase in intensity. Wortmannin, a drug that inhibits late endosome fusion, is used to block the transport of LDL to the lysosome. Individual dequenching events are not observed in wortmannin-treated cells suggesting that degradation of the LDL particle requires late endosome-lysosome interactions. This LDL labeling scheme will be extended to two-color single particle tracking experiments to determine the fraction of late endosome-lysosome interactions that lead to enzymatic degradation of LDL particles.

172-Pos Board B51

Activation Pathway Of paGFP In Living Cells

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Particle tracking inside the cell largely benefits of the ability to spatially and temporally mark specific structures to follow their ''signalling'' over a ''dark'' background as made possible since the advent of the photo-activatable markers. In terms of spatial confinement of the photo-activation process, the use of multiphoton excitation provides several favourable aspects compared to single photon confocal microscopy in photomarking biological structures to be tracked: the confined excitation volumes, of the order of magnitude of subfemtoliter, due to the non-linear requirements provide a unique control of the excitation and consequently photoactivation in the 3D space. In this context photoactivation experiments can be used to assess quantitative information about the binding kinetics of a macromolecule expressed in different cellular compartments. In this work we extended to photoactivation procedures and models originally developed for the quantitative analysis of FRAP experiments and we evaluated, for different proteins of medical interest (Rac-paGFP), the diffusive behaviour in the cytoplasm and the binding kinetics at the large endosomes. The results are compared with standard photobleaching experiments, in order to evidence the gained sensitivity obtained with photo-activatable proteins.

173-Pos Board B52

Organelle Specific Associations of HIV-1 Nef with HLA-I A2 and CD4 using Fluorescence Cross Correlation Spectroscopy

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Among HIV encoded proteins, ***Nef*** is critically required for virus replication and AIDS pathogenesis and is a major effector of immune-evasion mechanisms, in part by modulating cell surface expression of CD4 and HLA-I receptors. Genetic and biochemical studies have suggested two different mechanisms for *Nef* induced downregulation of CD4 and HLA-I. This might reflect distinct and differential interactions between *Nef* and the receptors in the various subcellular organelles. To evaluate this possibility, we measured the interactions of *Nef* -cerulean with HLA-I A2-eYFP protein in the TGN, ER and plasma membrane in transiently transfected HeLa cells using single point Fluorescence Cross Correlation Spectroscopy (FCCS). We found that between 27 to 37% of the total concentration of *Nef* was found to be associated with HLA-I A2 in these organelles.

We also studied the interactions of a CD4-eYFP on the plasma membrane with *Nef*-cerulean. We found that most of the *Nef* present in the plasma membrane binds CD4. Two diffusion components for CD4 were observed, with most of the binding between CD4 and *Nef* occurring in the more mobile fraction. Most of the *Nef* at the plasma membrane was also found to bind CD4 LL/AA mutant, which is not downregulated by *Nef* efficiently and thus was thought to bind *Nef* less avidly. We also demonstrate that removal of cholesterol by β-methyl cyclodextrin abolished the larger complexes.

174-Pos Board B53

Raster Image Correlation Spectroscopy (RICS) with One Photon Excitation and Analog Detection: Some Practical Considerations for GUVs and Cell Membranes

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Raster Image Correlation Spectroscopy (RICS) allows for mapping the local translational diffusion coefficient(s). The applicability of the technique has recently been extended by implementation on confocal laser scanning microscopes (CLSM) having one-photon laser excitation and analog detection [1-4]. To better understand the reproducibility and accuracy of RICS analysis of cellular membranes and the top membranes of Giant Unilamellar Vesicles (GUVs) [4], the influence and the constraints imposed by instrumentation characteristics and by sample properties on the retrieved diffusion values have been simulated. Similarly to Brown et al [1], particle numbers in the observation volume and analysis brick size were varied. In addition the workable scan speed range was explored as well as the amount of allowed detection noise. We present evidence for a drop in D values when mapping towards the GUV perimeter and corroborate results when both the total number of particles and the mapping brick size get small. Experimental results show that the magnitude of correlated detection noise in our Zeiss LSM 510 META confocal system (build 2002) along the fast x-scan axis ($\psi = 0$) is considerably larger than reported by others [1-3]. The effect of omitting the $\psi = 0$ line in the analysis was investigated. Both the LFD, UCI RICS package as well as our Matlab software based on routines supplied by D. Kolin (McGill University) gave similar simulation and translational diffusion mapping results.

[1] Brown 2008, J. Microsc. *229*, 78-91.
[2] Dalal 2008, Microscopy Research and Technique *71*, 69-81.
[3] Sanabria 2008, Biophys J doi:10.1529/biophysj.108.138974.
[4] Gielen 2008, J. Fluoresc. *18*, 813-819.

175-Pos Board B54

Effects of Oversampling and Scanning Artifacts on the Accuracy of Spatial Fluorescence Intensity Fluctuation Analysis Methods

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We present a systematic simulation and experimental study of potential measurement artifacts that arise due to raster scan acquisition for the image fluctuation analysis methods image correlation spectroscopy (ICS) and spatial intensity distribution analysis (SpIDA). With computer simulations, we show that photobleaching occurring during the sampling of sequential pixels affects the density and quantal brightness measurements obtained from single laser scanning microscopy images. This effect is even more pronounced when the images are oversampled with by using a small pixel size. The magnitude of this artifact was a function of the bleaching coefficients. The simulation results were compared to actual experimental data collected using a confocal laser-scanning microscope for a variety of dyes and sampling conditions. We analyzed images of monomeric fluorescent dyes covalently bound to coverslips, and monomeric green fluorescent protein (EGFP) transfected in mammalian cells. Moreover, we investigated the effects of non-constant emission of fluorescent probes, the presence of background noise and shot noise on the accuracy of the image fluctuation methods. We found that as the laser power increases (below saturation), some asymmetry in the spatial autocorrelation function appears which leads to systematic errors in the ICS measured number densities. SpIDA is also affected in the same manner by this artifact. Finally we looked at the accuracy of both techniques as a function of the quantal brightness of the particles. We generated computer simulated images of point emitters with different quantal brightness and then added shot noise to the images to determine accuracy and precision ranges for the measurements.

176-Pos Board B55

Biocompatible Quantum Dots for Intravital Kidney Imaging

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Quantum dots (QDs) are emerging alternatives to traditional dyes for fluorescence imaging applications. Quantum dots are attractive due to their photostability, broad excitation, narrow emission bands, high quantum yields and relatively long fluorescence lifetimes for contrast enhancement. We are interested in applying quantum dots (CdSe/ZnS) with biocompatible coatings for functional imaging of the kidney in vivo. Towards that end, we carried out experiments to characterize the bio- and photo-physical properties of CdSe/ZnS quantum dots with lipopolymer and aminoethoxy ethanol (AEE) coatings, and in vivo imaging of the kidney using surface coated quantum dots. We found that the fluorescence of the CdSe/ZnS quantum dots with surface coatings is enhanced in the presence of blood serum and it is serum concentration dependent. In addition, these surface coated quantum dots exhibit photo-induced fluorescence enhancement (PFE) and the PFE varies with specific surface coatings. Previously, PFE phenomenon is observed in CdSe quantum dots without surface coatings and has been studied under a number of different experimental conditions. To our knowledge, PFE of quantum dots with a surface coating has not been reported in the literature. For imaging application, we have