fraction for such *adapted cells* varies from phi = 0.16 at 0.10 Osm to phi = 0.36 at 1.45 Osm. For cells grown at 0.28 Osm, a similar range of is obtained by *plasmolysis* (sudden osmotic upshift) using NaCl as the external osmolyte, after which the cellular response is passive loss of cytoplasmic water. Using fluorescence recovery after photobleaching (FRAP), we measure the effective axial diffusion coefficient D_{GFP} of green fluorescent protein in the cytoplasm of live *E. coli* cells as a function of for both plasmolyzed and adapted cells. For *adapted* cells the median diffusion coefficient $D_{GFP}{}^{m}$ decreases by only a factor of 2.1 as phi increases from 0.16 to 0.36. In sharp contrast, for *plasmolyzed* cells $D_{GFP}{}^{m}$ decreases by a factor of 70 as phi increases from 0.16 to 0.33. Clearly GFP diffusion is not determined by phi alone. By comparison with quantitative models, we show that the plasmolysis data cannot be explained by simple crowding theory in a homogeneous medium. We will also report on measurements of time-resolved fluorescence anisotropy of GFP in the cytoplasm, diffusion of RNA polymerase in the cytoplasm, and diffusion of GFP in the periplasm of *E. coli.* Time lapse measurements monitor the recovery of cell volume and GFP diffusion after plasmolysis, which may be a key determinant of the time scale of the recovery of growth.

156-Pos Board B35

Measuring the Number of LuxR Proteins in a Single Cell of *V. harveyi*

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We have determined the number *N* of LuxR proteins in a single cell of the bacterium *V. harveyi* by measuring the distribution functions of cell volumes and protein-fluorescence intensities during cell division. In quorum sensing, the LuxR protein population, which regulates many (~70) genes, is sensitive to the concentration of auto-inducer molecules (AIs). We utilized a strain that is incapable of producing AIs. The LuxR proteins are tagged by a red fluorescent protein (mCherry). In the absence of AIs, the cells maintain a baseline residual concentration of LuxR that is remarkably constant over 8-10 cycles of cell division. We recorded the growth of a single cell into a large colony by imaging both phase contrast and mCherry intensity every 2 minutes in a 6-hour movie. The phase-contrast image was used to measure the volume of each cell, while the mCherry intensity monitored the LuxR population. At each cell-division event, we determined the fractional partitioning of the cell volume and the LuxR population. From the large number of cell-division events (~300), we obtained the normalized distributions of both the volumes and the mCherry intensities. Our procedure allows an accurate measurement of the width of the volume distribution ($\sigma = 0.031 \pm 0.003$). Significantly, the width of the LuxR distribution was observed to be much broader, presumably because of small-number fluctuations. By deconvoluting the Gaussian distributions, we find that the average LuxR copy number *N* equals 140 ± 10 just before cell division. Repeating the experiment at successively higher levels of applied AI concentration, we confirmed that when *N* is 10 times larger, the 2 distributions converge to the same width σ. This technique may be applied quite generally to other systems.

157-Pos Board B36

Influence Of P-selectin Structure On Its Mobility In The Weibel-Palade Body And Plasma Membranes

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The leukocyte adhesion molecule P-selectin is stored in Weibel-Palade bodies (WPBs), a secretory organelle of endothelial cells. The extracellular domain of P-Selectin comprises 9 consensus repeats (CRs), an EGF domain (E) and a Lectin domain (L) at the N-terminus, forming a rod-like structure approximately 48nm in length. Although truncation of extracellular CRs of P-Selectin impairs leukocyte capture under flow conditions, how such modifications affect the mobility of P-Selectin in the WPB membrane and in the plasma membrane (PM) after exocytosis is not known. Using single WPB FRAP or TIRFM with single fluorophore (SF) detection and tracking the diffusion of P-Selectin-EGFP and N- and C-terminal truncations of P-Selectin-EGFP was investigated in WPB or PM during ionomycin (1 μM) -evoked WPB exocytosis at 37°C.

P-Selectin-EGFP was immobile in the WPB membrane, but its N-terminal truncations rendered it mobile. On exocytosis SFs of P-Selectin-EGFP and its mutations were found to diffuse approximately freely in the PM in the vicinity of WPB fusion sites. The diffusion coefficient ***D*** for P-Selectin-EGFP was 0.14 $\mu m^2/s$, (n=2890 SF). Deletion of 8 of the 9 CRs increased ***D*** to 0.18 $\mu m^2/s$, (n=3907 SF). Removal of the L domain alone increased ***D*** to 0.24 $\mu m^2/s$, (n=1716 SF). Deleting both L and 8CRs increased ***D*** to 0.29 $\mu m^2/s$ (n=1818 SF). Removing E had no effect. C-terminal truncation also altered ***D***. The structure of P-Selectin influences its mobility in the WPB and PM.

158-Pos Board B37

Quantitative Analysis of Spatial Protein-protein Proximity in Fluorescence Confocal Microscopy

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Colocalization between fluorescently-labeled proteins has turned to be a measure of protein-protein interactions and a tool in cell biology. However, its evaluation has inherent caveats. The popular overlay method is qualitative and greatly depends on user setting for threshold values. Quantitative methods are also available, but the results can be unreliable because of the questionable assumption that proteins are uniformly distributed, and of the failure to minimize the influence of nonspecific labeling and random fluorescence noise. In order to quantify colocalization in a more absolute manner, we extended the use of image cross-correlation spectroscopy (ICCS) 1 to minimize the effect of protein distribution, non-specific labeling and random noise. The numerical procedure to separate the fluorescent components is based on the fact that the crosscorrelation and autocorrelation image values as function of x,y pixel shift have a peak at zero pixel shift decaying with sharp and shallow components as a function of x and y pixel shift. The sharp component corresponds to the co-localized proteins while the shallower one corresponds to non-specific labeling. By fitting the sharp and shallow landscapes of the crosscorrelation and autocorrelation functions to the sum of two Gaussian distributions, one can extract the peak amplitude of the specific sharp components to calculate the protein proximity index (PPI) from the ratio between the crosscorrelation and autocorrelation values at x,y=0 pixel shift. In summary, our method extracts the colocalization value from background generating consistent results from both computer simulated images and biological confocal images. Thus, it is a powerful microscopy tool to determine the nature of macromolecular complexes and their dynamic changes in biological processes.

1. Comeau JW, Costantino S, Wiseman PW. A guide to accurate fluorescence microscopy colocalization measurements. Biophys J. 2006;91:4611-22.

159-Pos Board B38

The White Confocal - Controlling Spectral Fluorescence

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Fluorescence has evolved to the most important tool in modern biological research. Specific histological stainings, antibody-based protein-markers and DNA-hybridization were the classical targets. Fluorescent proteins and other advanced stainings allow tracing molecules and structures in living samples - both by classical imaging as well as by modern analytical approaches; e.g. fluorescence correlation and its derivatives.

A very beneficial phenomenon in fluorescence is the fact that it comes in an infinite number of colors - which is at the same moment the most challenging feature. Multiple colors are available simultaneously - but at the price of a very elaborate illumination scheme, tricky beam splitting and efficient but selective detection of the various colors which are used to stain different structural elements in the sample.

All three modules, that are required for incident light fluorescence measurement instruments, are now spectrally tunable: white laser light sources, programmable acousto optical beam splitters and tunable multi-band emission detectors. These tunable elements allow for any spectral combination both on the excitation and the emission side. Reduction of crosstalk, more specificity and new measurements like excitation-emission correlation are some of the benefits of these developments. And as a side-effect: the acousto-optical devices transmit much better as compared to commonly used filters and dichroics.

Here, new approaches to use spectral information of both emission and excitation are presented and examples are given.

160-Pos Board B39

Identifying Components Of Astroglial Autofluorescence Using The Spectral Separability Index, Xijk

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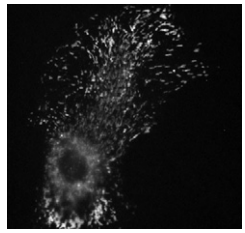
In in multi-color fluorescence, endogenous fluorophores have been considered more of a nuisance than a signal. Many of them co-exist (e.g., mitochondrial NADH and flavins) or, as ceroids and lipofuscins, have intrinsically broad fluorescence excitation and emission spectra. Thus, the presence of autofluorescence, along with cross-excitation and fluorescence bleed-through of one color channel into the neighboring one, bring up the question to which extent different color channels contain truly independent fluorophore information.

Here, we use a rigorously defined spectral separability index that combines the absorption and emission characteristics of fluorophore j with the spectral properties of the light source, excitation and emission filter(s), dichroic mirror and detector into one figure of merit that quantifies the amount of cross-talk in both excitation (i) and emission (k) channels. We used Xijk to detect in mouse cortical astrocytes two exogenous fluorophores (EGFP and Texas Red) in front of a multi-component autofluorescent background comprising at least three different components. We believe that Xijk offers a valuable tool to experimenters and reviewers for choosing suitable recording conditions and for evaluating and comparing co-localization, FRET and photoswitching data across set-ups and publications.



161-Pos Board B40

Combinatorial Labeling And Spectral Imaging, (CLASI): A Method To Greatly Expand The Number of Distinguishable Fluorescent Labels in a Single Image.

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The number of fluorescent proteins, organic fluorophores, and inorganic fluorescent biomarkers is ever increasing. However, the ability to unambiguously distinguish more than a few different labels in a single fluorescence image is severely hampered by the excitation cross-talk and signal bleed-through of fluorophores with highly overlapping excitation and emission spectra. Here, we report the development of a fluorescence labeling, imaging, and analysis method to greatly expand the number of identifiable labels in a single image. The CLASI method involves labeling targets with specific combinations of fluorophore reporters. Commercially available microscopes with spectral detection capabilities are used to image the combinatorially-labeled specimens. Novel computational algorithms are used to analyze spectrally-recorded image data. We have developed a linear unmixing algorithm constrained to identify specific combinations of fluorophores. Our novel algorithms allow the concatenation of spectral data acquired with several different excitation wavelengths, either in parallel or sequentially. A goodness-of-fit is reported for each spectral combination, either in every pixel or for every particle identified in the image. We have applied the CLASI method to the study of the composition and spatial arrangement of complex microbial communities. Using fluorescence in situ hybridization with oligonucleotide probes specific for 16S rRNA sequences, we demonstrate that we can distinguish 120 differently labeled microbes in a mixture using binary combinations of 16 fluorophores.

162-Pos Board B41

Blind Source Separation Techniques For The Decomposition Of Multiply Labeled Fluorescence Images

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Methods of blind source separation are used in many contexts to separate composite data sets into their sources. Multiply labeled fluorescence microscopy images represent such sets, in which the sources are the individual labels. The label distributions are the quantities of interest and have to be extracted from the images. This is often challenging since the effective emission spectra of fluorescent dyes are environment and instrument specific.

We developed a non-negative matrix factorization (NMF) algorithm to detect and separate spectrally distinct components of multiply labeled fluorescence images. It operates on spectrally resolved images and delivers both the emission spectra of the identified components and images of their abundances. We tested the proposed method using biological samples labeled with up to 4 spectrally overlapping fluorescent labels. In most cases, NMF accurately decomposed the images into the contributions of individual dyes. However, the solutions are not unique, when spectra overlap strongly or else when images are diffuse in their structure. To arrive at satisfactory results in such cases, we extended NMF to incorporate preexisting qualitative knowledge about spectra and label distributions. We show how data acquired through excitations at two or three different wavelengths can be integrated and that multiple excitations greatly facilitate the decomposition.

By allowing reliable decomposition in cases, where the spectra of the individual labels are not or only inaccurately known, the proposed algorithms greatly extend the range of questions that can be addressed with quantitative microscopy.

163-Pos Board B42

A Theory Facilitating the Investigation of Sub-resolution Membrane Trafficking Using Total Internal Reflection Fluorescence Microscopy

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Total internal reflection fluorescence (TIRF) microscopy has being used to investigate membrane structures smaller than optical resolution. Thus, a fluorescence punctum in a TIRF image reflects one or more such fluorescently labeled entities. We have developed a theory linking the fluorescence puncta to their underlying randomly distributed sub-resolution structures. This theory is verified using realistically simulated TIRF images of GLUT4 glucose transporters in cultured adipocytes, upon which fluorescence puncta are automatically identified using our MAX2D algorithm. We found the maximum puncta density in a TIRF image is directly limited by optical resolution, which is predicted and precisely described by our theoretical formulation. Within the limit of microscope's resolution, >90% true-positive rates are achieved for localizing an underlying sub-resolution vesicle to an identified punctum pixel location. Importantly, ~30-60% of all puncta locations are super-resolution (100nm) markers to their underlying sub-resolution structures. With the average inter-vesicle distances become much smaller than microscope's resolution at higher simulated vesicle densities, the closest matching vesicles to puncta locations are typically of high signal-to-noise characteristics, which in the TIRF evanescence field are also vesicles localized closer to the coverslip-attached plasma membrane where vesicle fusion occurs. We have extended our method to much smaller (synaptic vesicles) and larger (insulin granules) membrane compartments. Analysis of experimentally acquired data suggests insulin reduces the pool of exocytic GLUT4 vesicles near the adipocyte plasma membrane.

164-Pos Board B43

Expanding The Applicability Of The Multi-photon Fluorescence Recovery After Photobleaching Technique In Vivo Using A New Convective Flow Model

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Multi-photon fluorescence recovery after photobleaching (MP-FRAP) is a well-established microscopy technique that is finding its way into a host of *in vivo* applications. In this poster, we present the derivation of a new fluorescence recovery model that explicitly accounts for the possibility of convective flows in a system. We test this ''flow'' model through both simulations and *in vitro* experimentation, and demonstrate the new model *in vivo*. Results from our work show that the flow model significantly improves the capabilities of MP-FRAP *in vivo*, by yielding an accurate value for the diffusion coefficient, even when significantly large flow velocities are present.

165-Pos Board B44

Diffusion and Exchange of Non-Integral Membrane Associated Fluorophores During Fluorescence Recovery After Photobleaching with the Confocal Laser Scanning Microscope: ROI Size Analysis of EGFP:Ras2 Plasma Membrane Diffusion in Saccharomyces cerevisiae

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Binding, lateral diffusion and exchange are fundamental dynamic processes involved in protein localization in cellular membranes. In this study, we developed numerical simulations of lateral diffusion of a fluorophore in a membrane with arbitrary bleach geometry and exchange of the fluorophore with cytosol during Fluorescence Recovery after Photobleaching (FRAP) experiments. Based on our model simulations, we designed and performed FRAP experiments with varying bleach region sizes on plasma-membrane localized EGFP:Ras2 in live yeast cells to investigate the mobility and the presence of any exchange processes operating in the time scale of our experiments. Model parameters estimated from a 1 micron x 1 micron bleach region-of-interest (ROI) size successfully predicted the 0.5 micron x 0.5 micron bleach ROI experiment without additional fitting. Successful prediction of the second experiment without data fitting shows the agreement of the experiment with the theory and excluded alternative models including both diffusion and binding, which were also tested against the experimental data. We also performed Fluorescence Correlation Spectroscopy (FCS) experiments as an independent method to measure the mobility of EGFP:Ras2. We show that simulation of FRAP experiments based on the mobilities and fluorophore fractions derived from FCS model fits enables the validation of the FCS model. The methods developed in