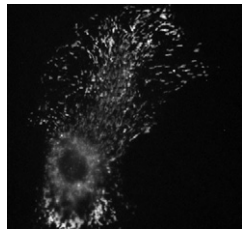
Here, we use a rigorously defined spectral separability index that combines the absorption and emission characteristics of fluorophore j with the spectral properties of the light source, excitation and emission filter(s), dichroic mirror and detector into one figure of merit that quantifies the amount 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.

Alex M. Valm[1,2], Jessica L. Mark Welch[1], Christopher W. Rieken[1], Yuko Hasegawa[1,2], Rudolf Oldenbourg[1,2], Gary G. Borisy[1].
[1]Marine Biological Laboratory, Woods Hole, MA, USA, [2]Brown University, Providence, RI, USA.

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

Richard A. Neher[1], Mišo Mitkovski[2], Frank Kirchhoff[2], Erwin Neher[3], Fabian J. Theis[4], André Zeug[5].
[1]UCSB, Santa Barbara, CA, USA, [2]Max-Planck Institute of Experimental Medicine, Goettingen, Germany, [3]Max-Planck Institute for Biophysical Chemistry, Goettingen, Germany, [4]Helmholtz Center, Munich, Germany, [5]University of Goettingen, Goettingen, Germany.

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

Shaohui Huang, Lawrence Lifshitz, Karl Bellve, Clive Standley, Kevin Fogarty, Michael Czech.
University of Massachusetts Medical School, Worcester, MA, USA.

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

Kelley D. Sullivan.
University of Rochester, Rochester, NY, USA.

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

Kalyan C. Vinnakota, David Mitchell, Robert J. Deschenes, Tetsuro Wakatsuki, Daniel A. Beard.
Medical College of Wisconsin, Milwaukee, WI, USA.

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

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

Benjamin P. Bratton, M.T. Record, James C. Weisshaar.
University of Wisconsin - Madison, Madison, WI, USA.

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

Laurent Holtzer[1], Anna Kicheva[2], Marcos Gonzalez-Gaitan[2], Thomas Schmidt[1].
[1]Leiden University, Leiden, Netherlands, [2]University of Geneva, Geneve, Switzerland.

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

Urszula P. Golebiewska[1,2], Suzanne Scarlata[1].
[1]Stony Brook University, Stony Brook, NY, USA, [2]Queensborough Community College, Bayside, NY, USA.

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

Robert Zorec[1,2], Matjaž Stenovec[1,2], Maja Potokar[1,2], Marko Kreft[1,2], Mateja Erdani Kreft[3].
[1]Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, [2]Celica Biomedical Center, Ljubljana, Slovenia, [3]Institute of Cell Biology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia.

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

Alex Robson[1], Marcus Tyndall[2], Philip Maini[1], **Mark C. Leake**[1].
[1]Oxford University, Oxford, United Kingdom, [2]Reading University, Reading, United Kingdom.

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

William H. Humphries IV, Christine K. Payne.
Georgia Institute of Technology, Atlanta, GA, USA.

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