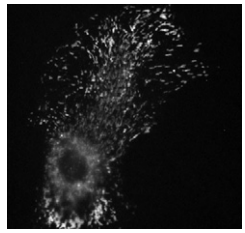
Here, we use a rigorously defined spectral separability index that combines the absorption and emission characteristics of fluorophore j with the spectral properties of the light source, excitation and emission filter(s), dichroic mirror and detector into one figure of merit that quantifies the amount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