iterations of random ion placement, implicit solvent molecular dynamics simulations and statistical analysis. Our resulting model matches very well with existing mutagenesis data, and subsequent explicit solvent molecular dynamics simulations have been performed using this Ca^{2+} bound structure. Comparison of the dynamics and conformations of the Ca^{2+} bound and unbound simulations reveal a concerted conformational change in the structure and suggest a potential mechanism for calcium dependent activation of these channels.

Imaging & Optical Microscopy II

2029-Pos
Characterization of the Use of Far-Red Dyes for Localization Microscopy of Biological Samples
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Recently it has been shown that conventional fluorescent dyes can be used to achieve super-resolution by single-molecule localization. The use of conventional fluorochromes for this new approach, termed 'localization microscopy', depends critically on the observation of rapid on-off blinking of these dyes in certain chemical environments. Here we characterize the photophysical behavior of several commercially available far-red dyes that have similar properties to the cyanine dye Cy5 and use them to visualize the distribution of proteins in fixed cardiac cells.
To obtain super-resolution images several thousand individual 'blinking'-events, which arise from dye molecules that briefly enter a bright (fluorescent) state, were analyzed and the molecular positions determined by a fitting algorithm. We tested far-red Alexa dyes (647,680,700,750) linked to secondary antibodies for use in immuno-labeling super-resolution microscopy. The dyes were observed in mounting media of various refractive indices containing oxygen scavengers and triplet quenching compounds that favors fluorochrome 'blinking'. Under these conditions typically over 1000 photons per 'blinking'-event were detected and a localization accuracy of ~30 nm (full width at half maximum) was readily achieved with Alexa 680. These accuracies are in good agreement with theoretical calculations when the background present in labeled cellular preparations (largely arising from out-of-focus fluorescence) is taken into account.
For practical super-resolution imaging of immuno-stained preparations, several advantages are provided by these dyes, for example, as compared to blue/green excited fluorochromes less autofluorescence is generally caused by organelles and fixation. We investigate the possibility to use a combination of these dyes for simultaneous localization imaging of multi-labeled preparations excited with a single laser line. This is illustrated using fixed ventricular myocytes that were labeled for β-tubulin, caveolin-3 and ryanodine receptors.

2030-Pos
Synthetic Chromophore Maturation by Split Green Fluorescent Protein (GFP)
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Green fluorescent protein (GFP) undergoes a self-catalyzed cyclization, dehydration, oxidation reaction sequence to form a fluorescent chromophore in the protein's interior. We have developed a system for studying chromophore maturation and the photophysical properties of novel chromophores *in vitro*. Intact GFPs, circularly permutated to locate the interior α-helix at the N-terminus, were expressed with a flexible proteolytic loop inserted on the C-terminal side of the chromophore-containing helix. With trypsin, the helix was severed and subsequently removed by size exclusion chromatography after denaturation. The split GFP was then renatured in the presence of a synthetic peptide which underwent chromophore maturation to acquire fluorescence. Since the complementary helix peptide is fully synthetic we have unprecedented control through specific incorporation of multiple unnatural amino acids. We are studying these effects with a range of spectroscopic techniques including steady state fluorescence, time-correlated and upconversion time-resolved fluorescence in order to better understand the process of chromophore maturation, the photochemistry of the protein, and the kinetics and efficiency of fluorescence reconstitution to inform the strategies for producing more robust *in vivo* probes.
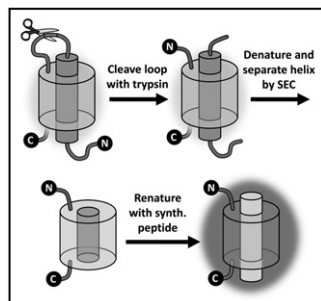
2031-Pos
Dynamics of Individual BK$_{Ca}$ Channels in Live Cells Monitored by Site-Specific Labeling Using Quantum Dots
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In order to monitor the movement of individual large-conductance Ca^{2+}-activated K^+ channels (BK$_{Ca}$ channels) in live cells at real-time, we co-expressed the BK$_{Ca}$ channel tagged at its extracellular N-terminus with the 'acceptor peptide (AP)' for biotin and a genetically modified *E. coli* biotin ligase in various mammalian cells. Using the quantum dots (QDs) coated with streptavidin, we were able to visualize individual BK$_{Ca}$ channels that had been biotinylated intracellularly and then expressed on to the surface of the cells. The channels were determined to be labeled by two QDs in average, based on the levels of quantized fluctuations of fluorescence intensity, known as 'photo-blinkings'. We monitored the movements of BK$_{Ca}$ channels in both live mammalian cell-lines and primary hippocampal neurons using time-lapse imaging. Depending on the type of cells and the location where the channels were expressed within a cell, BK$_{Ca}$ channels showed different patterns and speeds in their movements. We are currently quantifying the movement of individual channels and investigating those protein motifs affecting the channel dynamics. We wish to understand the molecular mechanism of BK$_{Ca}$ channel trafficking and the cellular players involved in.

2032-Pos
Counting Pictures of Cell that Count: A Biotiff-Based Strategy for Indexing Cell Images and Associated Metadata in Large Series of Digital Microscopy-Based Biophysical Result
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Last year we described how BioTIFF code (www.biotiff.org) enables all metadata needed to interpret a given digital image region to be embedded within the same file structure used to store that image (Biophys J. 96: 30a). Here we show how that metadata can then be indexed so that Google-like queries can be performed on any set of BioTIFF files. Indexing can be done either on a single local set of BioTIFF images or a distributed set by using a distributed file system. This then creates a distributed index that has many interesting applications for exploring image relationships across different subsets of images within large sets. For example, single cell (or single molecule) responses measured using digital microscopy-based biophysical methods that relate cellular mechanism to light-based surrogate measures can then be linked to a specific cell in a specific field-of-view of a specific sample. The replication of such results over time in the same lab or in a distributed manner by multiple labs can allow for collaborative replicable science to anchor the evolution of shared understanding of cell physiology mechanisms. We will demonstrate how the approach can be implemented using a plurality of commodity gear meeting certain minimum standards and open source imaging, indexing and search software. This level of transparency and annotation of experimental detail allows for differences in experimental conditions between experiments and labs to be accommodated in open collaborative interpretation of biophysical data.

2033-Pos
Site-Specific, Orthogonal Two Color Labeling of Different Proteins with Flash and Reash in Living Cells
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Multiparameter imaging of independent cellular functions is limitied by the lack of distinct molecular probes that would specifically label two different proteins. Here we report a strategy to simultaneously label two different proteins in living cells with two different fluorophores, FlAsH and ReAsH.
Recently, tetracysteine binding motifs have been improved to selectively bind to FlAsH or ReAsH respectively. We compared the six amino acid motif CCPGCC and the twelve amino acid motif FLNCCPGCCMEP with respect to their affinities for FlAsH and ReAsH. Both fluorophores showed higher affinity for the FLNCCPGCCMEP motif. For both target sequences, FlAsH showed more stable interactions than ReAsH. Using a new labelling protocol we selectively labeled different proteins in the same cell. Our target proteins were localized in different cellular compartments, a plasmamembrane localized G protein-coupled receptor for PTH (PTH-receptor) and the cytosolic β-arrestin2- protein. Our protocol allowed selective labelling of PTH-receptor with ReAsH at a C-terminal FLNCCPGCCMEP motif, while the cytosolic β-arrestin-2 protein was C-terminally modified with the CCPGCC motif and specifically labeled with FlAsH. Both proteins were simultaneously visualized in intact cells, and their interaction was determined by colocalization and fluorescence resonance energy transfer (FRET).
Taken together, our data demonstrate that FlAsH and ReAsH can be used for orthogonal labeling of different target proteins in living cells.