Orai3-like gating characteristics, in a strongly cooperative manner. In conclusion Orai subtype-specific gating requires a cooperative interplay of all three cytosolic domains.

1000-MiniSymp
Molecular Determinants of CRAC Channel Gating
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Store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channels regulate numerous cellular functions including transcription, motility, and proliferation in many cell types. The discoveries of Orai1, the CRAC channel pore subunit, and STIM1, the ER Ca^{2+} sensor, have produced rapid progress in our understanding of the molecular features of the CRAC channel pore and the cellular events involved in channel activation. It is now known that following depletion of intracellular Ca^{2+} stores, STIM1 activates CRAC channels by interacting with the cytoplasmic N- and C-terminal domains of Orai1. Orai1 and Orai3 CRAC channels can additionally be activated in a store-independent fashion by the compound 2-APB. However, the molecular and structural mechanisms of STIM1- and 2-APB-mediated gating remain poorly understood. Using a combination of site-directed mutagenesis and cysteine accessibility analysis, we are probing the structural alterations in the pore that occur during STIM1- or 2-APB-mediated activation of Orai1 and Orai3 channels. Our data indicate that the pore of the CRAC channel changes significantly as it transitions from the closed to the open state. Specifically, STIM1 binding causes large modification of the pore architecture, giving rise to features classically associated with CRAC channels such as its narrow pore and high Ca^{2+} selectivity. Thus, in addition to serving as the ER Ca^{2+} sensor and activator of the CRAC channel, STIM1 appears to function as a channel subunit, modifying the structural features of the pore to confer its unique permeation profile in the active state.

1001-MiniSymp
Activation of Orai1 Channels by Mutation of a Conserved Glycine Residue in TM1
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Stim and Orai proteins comprise the molecular machinery of Ca^{2+} release-activated Ca^{2+} (CRAC) channels. In T-lymphocytes, Orai1 is the pore-forming subunit and STIM1 serves as the ER Ca^{2+}-sensor that opens Orai1 following ER Ca^{2+}-store depletion. We are investigating mutations within Orai1 TM1 that alter channel activity in transfected HEK cells. At the extracellular side of TM1, we and others had previously shown that E106 confers Ca^{2+} selectivity on the STIM1-operated Orai1 current. We now show that R91, the site of the R91W mutation that causes severe combined immune deficiency, forms a very narrow part of the conducting pore at the cytosolic side of TM1. Orai1 R91C when co-expressed with STIM1 was activated normally by Ca^{2+} store depletion. However, treatment with diamide, a thiol-oxidizing agent, induced formation of disulfide bonds between R91C residues in adjacent Orai1 subunits. Moreover, diamide rapidly blocked STIM1-operated Ca^{2+} current, and current recovered during treatment with a reducing agent. In the middle of TM1, mutation of a conserved glycine residue to alanine (G98A) prevented STIM1-induced channel activity. Interestingly, mutation to aspartate (G98D) caused constitutive channel activation in a STIM1-independent manner to form a non-selective Ca^{2+}-permeable conductance that was relatively resistant to block by Gd^{3+} (310 nM K_d vs 7 nM in wild-type Orai1). Moreover, the double mutant R91WG98D was also constitutively active, overcoming the normal inhibition of channel activity by tryptophan at the 91 position; and the double mutant R91CG98D was resistant to diamide block. We tentatively propose that R91 forms the gate of the Orai1 channel at the narrow inner mouth of the channel, G98 functions as a gating-hinge, and E106 promotes selective conduction of Ca^{2+}. The channel pore is widened and ion selectivity perturbed by a negative charge at the G98 site.

PLATFORM W: Imaging & Optical Microscopy I

1002-Plat
Circular Scanning Fluorescence Correlation Microscopy on Membranes
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In circular scanning Fluorescence Correlation Spectroscopy (FCS) the measurement volume is scanned in a circle with a sub-micron radius, allowing determination of the diffusion coefficient and concentration without any a priory knowledge of the size of the detection volume. This feature is particularly important in measurements on two-dimensional surfaces, where the volume size, and therefore the quantitative outcome of the experiment, is determined by the relative position of the surface and the objective focus, a parameter difficult to control in practice. We have implemented this technique in a simple instrument based on a 2D piezo scanner, and applied it to studies of molecular diffusion in model systems, supported lipid bilayers and giant unilamellar vesicles, and in living cells. The method is shown to be minimally sensitive to disturbing interferences due to fast kinetics (dye photophysics) and long-time effects (axial membrane motion, depletion due to photobleaching), and yields reliable results even for tilted or fluctuating membranes. The resulting robustness makes this technique particularly suitable to applications in living organisms.

1003-Plat
Probing T-Tubular Electrophysiology by Random Access Two-Photon Microscopy in Cardiac Myocytes
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In cardiac cells many membrane channels are heterogeneously distributed between Surface and T-tubule membranes. Simultaneous recording of membrane potential in the two sarcolemma domains can reveal potential peculiarities of T-tubule electrophysiology. This is not feasible with traditional electrophysiological techniques. Current optical techniques for recording membrane potential can potentially overcome the microelectrode limitation. However, most approaches to the optical recording of membrane potential events lack the spatial and temporal resolution needed for regional assessment of action potential (AP) profile. Here, we developed an ultrafast random access two-photon microscope capable of optically recording fast membrane potential transients in multiple positions of the cell membrane with μm spatial resolution. The random access microscope, in combination with a novel voltage sensitive dye, was used to simultaneously record AP in surface sarcolemma and T-tubules in isolated cardiac myocytes with sub-millisecond time resolution. We found that in myocytes, paced at 0.2 Hz, the AP in the T-tubule has identical amplitude and kinetics as in the surface sarcolemma, indicating that the tight electrical coupling between the two membrane domains prevails over the inhomogeneous distribution of membrane currents. Consistently, in myocytes that had been acutely detubulated by formamide-induced osmotic shock, T-tubule AP was absent, indicating a complete uncoupling from the surface sarcolemma. The electrophysiological properties of t-tubules may be altered in pathological conditions, when detubulation and T-tubule remodelling occur. To mimic a model of pathological detubulation, myocytes were cultured for 24-36 hours, thus obtaining a significant loss and disorganization of the T-tubular network. Membrane staining confirmed the loss and morphological alterations of T-tubules; however, the electrical activity in the remaining remodelled T-tubules was preserved, suggesting that remodelled T-tubules were still coupled to the surface sarcolemma.

1004-Plat
Fluorescence Imaging of Influenza Virus H1N1 mRNA in Living Infected Cells using Single Chromophore FIT-PNA
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Replication of the influenza virus involves, amongst other critical steps, the synthesis of viral mRNA which is used for ribosomal synthesis of viral proteins. Significant efforts have been devoted to the development of methods that allow the imaging of specific mRNA sequences in living cells. In contrast to other approaches, fluorescent oligonucleotide probes enable the analysis of non-modified wild-type targets. For imaging the probes must recognize the target with high specificity and deliver measurable signals that provide high signal-to-background ratios. We have introduced single labelled peptide nucleic acid (PNA) probes, so-called FIT-PNA probes, which contain a single thiazole orange intercalator that serves as artificial fluorescent nucleobase. These probes respond to changes of the local structure in the vicinity of the dye rather than to the more global changes of conformation that are required for fluorescence signalling by the dual labelled molecular beacons. FIT-probes are unique because a single fluorophore provides for both high sensitivity and high target specificity at non-stringent hybridization conditions where both matched and mismatched probe-target complexes coexist. We show that FIT-probes are ideally suited for applications in live cell RNA imaging. We chose mRNA coding for neuraminidase of influenza virus A/PR/8 as a target. FIT-PNA probes are useful to detect the expression of viral mRNA in single living infected cells with high specificity. In particular, FIT-PNA sensitivity was superior to that of a molecular beacon. Our study suggests that FIT-PNAs are attractive tools

to study gene regulation of processes in living cells. The approach can be readily applied to other viruses as well.

1005-Plat

Quantitative TCSPC FRET-FLIM Applied to Donors Exhibiting Multi-Exponential Decays: Spatio-Temporal Interaction Between Calmodulin and the Kv7.2 Potassium Channel in Living Cells

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The spatio-temporal study of the dynamics of molecular interactions using FRET-FLIM is generally compromised by the large number of photons required to fit the multiple-lifetime decay of the donor population in each pixel of an image. Long acquisitions prevent interacting dynamics to be detected in an image, while the use of high excitation intensities results in artifactual measurements due to bleaching. The computation of the minimal fraction of donor molecules (mf_D) undergoing FRET allows quantitative imaging of molecular interactions with either single or multi-lifetime donors such as CFP, where the complexity associated to fitting a fluorescent decay with more than two components hampers quantification with traditional least-squares fitting strategies. This novel non-fitting analysis has been recently applied to wide-field time gated FLIM systems and we now extend it to more widely available TCSPC systems.

The use of mf_D analysis has allowed us to study the spatio-temporal dynamics of the interaction between CFP-tagged Kv7.2 channels (donor) and YFP-labeled calmodulin (acceptor) in HEK293 living cells on a TCSPC system. We show the existence of discrete interacting domains that can be followed as a function of time (every 12-15s), where the fraction of interacting KCNQ2 with calmodulin oscillates at least between 15% and 25%.

In summary, we have shown that the mf_D analysis allows quantitative study of the spatio-temporal dynamics of molecular interactions on TCSPC systems. Using this non-fitting strategy we have quantitatively imaged the interacting dynamics of CFP-tagged Kv7.2 channels and YFP-labeled calmodulin in living cells.

1006-Plat

N-Way FRET Microscopy for Imaging Multiple Protein Interactions Within a Single Living Cell

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Fluorescence Resonance Energy Transfer (FRET) microscopy has emerged as a powerful tool for probing nanoscale protein interactions while capturing the microscale organization of these interactions. However, current FRET microscopy approaches are limited to the analysis two interacting fluorescently labeled proteins at a time. This limitation precludes the use of FRET microscopy for simultaneous measurement of multiple biochemical activities. Here we present a new FRET microscopy method that generalizes quantitative FRET microscopy to any number of fluorophores interacting in any combination. This approach makes use of Parallel Factor Analysis (PARAFAC) to define excitation/emission spectral fingerprints for FRET between any number of interacting fluorophores from observations of reference samples on any instrument. The resultant spectral fingerprints are then used in a simple linear unmixing model recover the distributions of free and interacting fluorophores as well as their apparent FRET efficiencies. Input data can consist of either complete spectral data or filter-based methods. The approach was validated using control constructs consisting of fluorescent protein fusions inside living cells. This method will enable intracellular analysis of sequential biochemical interactions that could not previously be observed.

1007-Plat

Single Cell Sensing and Manipulation by Scanning Nanopore Microscopy

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Signal Transduction by Ion Nano-Gating (STING) technology is a label-free biosensor based on quartz nanopipettes capable of detecting DNA and proteins. Using conical quartz nanopipettes a Scanning Ion Conductance Microscope (SICM) was developed and used for single cell imaging, sensing and manipulation. The height of the nanopore above a surface can be accurately controlled by measuring the ionic current through the pore as it approaches a surface. By maintaining a predefined distance from a surface and raster scanning the pore, a topographical image can be acquired. Additionally, material can be ejected through nanopipette using electrophoretic and electroosmotic properties. The cost of fabrication and ease of use of nanopipettes provides an ideal system for developing cellular systems for both cell detection and control. We demonstrate the capability of this sensor to image living cells, deposit material for controlled single cell growth on a substrate, and a platform for single cell injection and detection using an integrated microfluidic chip. The combination of STING technology with traditional SICM technology can be leveraged to acquire both topographical data and functional data, such as local ion concentration. This form of complementary microscopy is henceforth termed functional Scanning Ion Conductance Microscopy (fSICM). Preliminary fSICM results will be presented. This work illustrates the applicability of the STING sensor to a variety of applications, from single cell detection to definition of cellular circuits.

1008-Plat

In Vivo Identification of Changes in Metabolic State as Stem Cells Differentiate, by Phasor Analysis of Fluorescence Lifetime Imaging

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In stem cell research there is a high demand of techniques to investigate self-renewal and differentiation mechanisms and to develop stem-cell-based therapies for regenerative medicine. Here we develop a label-free method to identify and classify stem cells and differentiating cells according to their metabolic state. We use the phasor approach to fluorescence lifetime imaging and intrinsic biochemical fluorescence biomarkers such as NADH, flavins, retinoids and porphyrin. The organ studied is the C. elegans germ line, expressing a histone-GFP fusion protein that allows us to identify the differentiation state of the germ cells. We calculate the average phasor value of the intrinsic fluorescence of germ cells and we plot the cell phasor fingerprints in a scatter diagram. Cell phasor fingerprints cluster according to their differentiation state. Different metabolic fingerprint of cells reflect changes in binding sites of NADH with different coenzymes during differentiation. The phasor approach to lifetime imaging provides a label-free, fit-free and sensitive method to identify different metabolic state of cells during differentiation, to sense small changes in the redox state of cells and may identify symmetric and asymmetric divisions and predict cell fate. This method is also a promising non-invasive optical tool for monitoring metabolic pathways during differentiation or disease progression, and for cell sorting.

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1009-Plat

Multiplexed Time Lapse Fluorescence Lifetime Readouts in an Optically Sectioning Time-Gated Imaging Microscope

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The aim of our study is to follow the temporal and spatial interactions of proteins within different signalling networks in live cells. To map protein-protein interactions, we apply Förster resonant energy transfer (FRET) readout using fluorescence lifetime imaging (FLIM) and we present an optically sectioned FLIM microscope capable of time-lapse readouts of multiplexed FRET pairs. We note that FLIM FRET provides robust measurements, permits the use of dark acceptors and helps minimise the impact of spectral cross-talk.

Wide-field FLIM is implemented using a gated optical intensifier synchronized with the excitation pulses from a fibre-laser pumped supercontinuum source or a frequency doubled Ti:Sapphire laser and enables FLIM FRET in live cells with acquisition times below ~10 seconds. Optical sectioning is implemented using a Nipkow disk unit and improves quantitation compared to wide-field imaging, e.g. permitting separation of signals from the plasma membrane and the cytosol.

To demonstrate the capabilities of this instrument, we have mapped the intracellular changes of calcium levels using interleaved time lapse FLIM acquisitions of live cells labelled with two calcium probes following stimulation with ionomycin and calcium solutions. HEK293 cells were labelled with the genetically expressed biosensor, Troponin TN-XL, for which changes in calcium levels are read out by FRET between the CFP and YFP fluorophores, and with a calcium sensing dye, GFP-Certified™ FluoForte™. The emission profile of the latter is spectrally separate from CFP and YFP, making it useful for multiplexing with the many available CFP/YFP FRET biosensors.

We aim to apply this instrument to follow the spatial and temporal changes of simultaneous activation of signalling pathways during fibroblast migration in a concentration gradient of a chemo-attractant (PDGF), particularly using specifically designed FRET biosensors for small GTPases (Rac), phosphoinositide pathway (IP3) and calcium.