with biotin-MHC/peptide to bind the TCR with relatively high avidity, followed by streptavidin-quantum dots. They were then imaged on an emCCD-equipped microscope and kICS analysis was applied. Spatial intensity fluctuations in an image measured the clustering of receptors on the 100s of nm length scale. Changes in the intensity correlation function of the blinking QDs characterized clustering on the 10s of nm length scale. We also used kICS to measure changes in TCR diffusive transport. When T cells exhibited maximum activity 3-4 days after exposure to antigen, the degree of their TCR aggregation on both length scales was significantly higher than that of naïve cells, while TCR diffusion was a minimum. This new technology has powerful applications as it can be applied to just a few cells and we will show that it is able to detect changes in receptor organization of cells in vivo.

3958-Plat
The Pair-Correlation Approach to FCS
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Molecular diffusion and transport processes are fundamental in physical, chemical, biochemical and biological systems. Current approaches to measure molecular transport in cells and tissues based on perturbation methods like fluorescence recovery after photobleaching are invasive, fluctuation correlation methods are local and single particle tracking requires the observation of isolated particles for relatively long periods of time. We propose to detect molecular transport by measuring the time cross-correlation of fluctuations at a pair of locations in the sample. When the points are further than two times the size of the point spread function, the maximum of the correlation is proportional to the average time a molecule takes to move from a specific location to another. We demonstrate the method with simulations, using beads in solution and by measuring the diffusion of molecules in cellular membranes. The spatial pair cross-correlation method detects barriers to diffusion and heterogeneity of diffusion because the time of the correlation maximum is delayed in the presence of diffusion barriers. This non-invasive sensitive technique follows the same molecule over a large area producing a map of molecular flow and does not require isolated molecules thereby many molecules can be labeled at the same time and within the point spread function. Work supported in part by U54 GM064346 Cell Migration Consortium (MD and EG), NIH-P41 P41-RRO3155 (EG) and P50-GM076516 (EG).

3959-Plat
Spatially Resolved Fluorescence Fluctuation Spectroscopy (FFS) in Living Cells
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Fluorescence (cross-)correlation spectroscopy (FCS/FCCS) and generally fluorescence fluctuation spectroscopy (FFS) are confocal microscopy-based methods that allow to assess diffusion and transport properties as well as interactions of molecules (proteins, nucleic acids, compounds) in vitro and in vivo. Commercially available instrumentation enables routine measurements at one or few specific points inside living cells.
However, conventional FCS/FCCS experiments remain challenging because point measurements in a living cell are associated with large error caused by the heterogeneous environment of the cellular interior. Moreover, biological noise due to cell-to-cell variations of physical and biological parameters (e.g. intracellular viscosity, protein expression levels) induces further variations, which are difficult to separate from the measurement error. Currently, these problems are partially addressed by performing statistical data analysis of measurements from many different cells. However, it is desirable to obtain more reliable and robust data from single cells with spatial resolution. This requires a new approach allowing to perform simultaneous measurements and to circumvent the problems associated with confocal FFS: photobleaching, out-of-focus illumination and loss of spatial definition due to cell movements.
Here, we present a novel microscope that allows spatially resolved FFS measurements in 2D optical sections across cells. The setup is based on a single plane illumination microscope in which a thin diffraction-limited light sheet is used to illuminate a cross-section of the cell. The use of an electron-multiplying charge-coupled device (EM-CCD), placed perpendicular to the light sheet, with hundreds of single pixel detectors instead of an avalanche photodiode (a single pixel detector) enables to record on each pixel the incoming photons with single photon sensitivity and sub-millisecond time resolution. This is predicted to significantly reduce the error associated with single point measurements. It should also provide access to spatially resolved measurements of concentrations, interactions and mobilities.

3960-Plat
Multiplexed Measurement of Molecular Interactions using Hyper-Spectral Imaging and Multi-Parametric Detection
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A large number of molecules cooperate in an intricate network of interactions for the maintenance of the structural integrity, the metabolism and the function of the living cell. A challenge for engineering and physics in optical microscopy is to provide tools that could offer the highest spatio-temporal resolution with the capability to decode complex networks of molecular interactions by the development of technologies and methods that, at the same time, may provide cost-effective and user-friendly instruments.
We present our latest development of a novel architecture for a spectrograph that permits to characterize fluorescence emission (excitation and emission spectra, fluorescence anisotropy and fluorescence lifetime) in a quantitative and efficient manner. The novel system offers parallel acquisition with a single detector and, by the use of a novel solid-state detector (time-gated single-photon avalanche photodiodes) and a supercontinuum light source, it provides excellent versatility of use at comparatively low costs. We envisage that by the exploitation of Foerster resonance energy transfer between a number of fluorophores, this microscopy platform will be capable to probe multi-molecular interactions and to multiplex a variety of fluorescent biosensors.
Novel biophysical imaging techniques are fundamental for our research activities in cancer research: to probe the key molecular processes underlying genomic stability and for a better understanding of the molecular aspects of cancer.

3961-Plat
Analysis of Cdc-42 Mobility and Dimerization In Vivo by Higher Order Fluorescence Correlation Cumulants
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We demonstrate a new method for measurements of mobility dependent protein oligomerization using higher order fluorescence correlation cumulants. Fluorescence intensity distribution methods including fluorescence cumulant and moment analysis have been successfully used in recent years to analyze oligomerization phenomena. The extension of such methods to treat analysis at different binning times allows for the analysis of mobility dependent oligomerization. Nevertheless, the analysis of time binned distributions is mathematically complex and depends strongly on detector characteristics such as afterpulsing and dead time. Here we develop an equivalent method treating traditional correlation functions as bivariate fluorescence cumulants. Doing so brings the power of the cumulant analysis to bear on the measurement of mobility dependent oligomerization while maintaining the mathematical simplicity of the correlation functions that are the standard within fluorescence correlation spectroscopy. We use this technique to show that the low mobility pool of intracellular EGFP-cdc-42 in living yeast cells is on average a dimer while the high mobility pool is monomeric. Examination of mutant yeast strains suggests that the low mobility pool is associated with recycling vesicles, providing an explanation for both the slow diffusion as well as the oligomeric state of this species. This technique could be easily extended to other proteins in the yeast genome that demonstrate heterogeneous mobility.

3962-Plat
H33D Gen II: A New Photon Counting Camera for Single-Molecule Imaging and Spectroscopy
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We have developed a new generation of photon-counting camera consisting of a large area microchannel plate-based photomultiplier tube associated with a position-sensing anode. Our detector can record local count rates of approx 30 kHz and sustain global count rates of several MHz. We illustrate its capabilities by tracking single quantum dots in live cells with nanometer spatial resolution and sub-ms temporal resolutions over large areas and long durations.

Additionally, the photon-counting nature of the detector allows spatial and temporal correlations to be obtained very easily. Finally, the detector contains an integrated TCSPC electronics that makes it a perfect tool for fluorescence lifetime imaging. This and other types of detectors currently in development will transform the way single-molecule imaging and spectroscopy experiments are designed and performed.

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3963-Plat
Light Sheet Microscopy Optimized for Depth Penetration to Study Embryogenesis
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Fluorescence light sheet microscopy (FLSM) has gained widespread recognition in recent years, due to its distinct advantages for the 3-dimensional (3D) imaging of living biological samples. FLSM uses a planar sheet of light to illuminate a sample, generating fluorescence over an optical section of the sample that is collected by a wide-field microscope camera oriented orthogonal to the light sheet. The orthogonal geometry between the illumination and detection pathways enables massive parallelization in both illumination and detection; futhermore, it permits optical and physical access to samples (3D cell cultures or whole embryos) in ways that are impossible in the collinear geometry of standard microscopes. Because of these features, FLSM significantly outperforms standard laser-scanning confocal microscopy in imaging speed, phototoxicity, and signal to noise in many imaging applications. An important aspect of any 3D imaging technique is its imaging depth limit (how deep into a sample useful information can be collected). In this respect, standard FLSM fares only slightly better than confocal microscopy. To overcome this hurdle, we have optimized FLSM for imaging of live thick samples by minimizing the degradation of the light sheet due to scattering, while preserving acceptable axial resolution. Using this approach we have imaged whole, live fruit fly embryos and zebrafish embryos. We achieve higher depth penetration than standard FLSM, while maintaining sub-cellular resolution, at imaging speed of about ten times faster than standard confocal microscopy.

Platform BJ: Member-Organized Session: Kinetics, Mechanisms & Regulation of Ion-Transporting ATPases

3964-Plat
Characterization of Partial Reactions in the Catalytic Cycles of Calcium and Copper ATPases
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Calcium and copper P-type ATPases transduce ATP chemical energy to transmembrane osmotic energy, using a catalytic mechanism common to haloacid dehalogenases where phosphoryl transfer from substrate to a conserved aspartate yields a phosphoenzyme intermediate before hydrolytic cleavage of Pi. Favored by its native abundance, characterization of the calcium ATPase is by now quite detailed, including two transmembrane calcium binding sites, a phosphorylation site within the headpiece, a conserved TGES motif for catalytic assistance of hydrolytic cleavage, and domain movements permitting long range linkage of phosphorylation and calcium binding sites. Presently, mutational analysis is still yielding further details on the calcium ATPase. As for bacterial (CopA) and human (ATP7B) copper ATPases, due to low native abundance, heterologous expression in cultured cells is required. In addition to domains present in other P-type ATPases, copper ATPase sequences include an amino terminus extension (NMBD) with one (CopA) or six (ATP7B) copper binding sites. Using recombinant protein, it is possible to demonstrate formation of phosphoenzyme intermediate by utilization of ATP, undergoing rather slow turnover. In analogy to the calcium ATPase, phosphoenzyme intermediate is not formed following mutation of the conserved aspartate or the transmembrane copper site. In addition to the phosphorylated ATPase intermediate, copper dependent phosphorylation of various serine residues occurs in ATP7B. Interference with protein autophosphorylation, both of aspartate and serines, is observed following mutation of a histidine residue in the nucleotide binding domain, a mutation found in Wilson disease of humans. The effects of NMBD copper site mutations and deletions suggest that the NMBD sequence is involved in catalytic regulation as well as protein targeting. (Supported by 5 R01 HL069830-08).

3965-Plat
The Involvement of Protein-Protein Interactions in the Mechanism of the Na^+,K^+-ATPase
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The Na^+,K^+-ATPase (or sodium pump) was the first ion pump to be discovered (Skou, 1957) and it is one of the most fundametally important enzymes of animal physiology. The electrochemical potential for Na^+, which the enzyme maintains, is used as the driving force for numerous secondary transport systems, e.g. voltage-sensitive Na^+ channels in nerve. ATP provides the energy source to drive ion pumping. However, it also plays a crucial allosteric role, accelerating significantly the enzyme's rate determining E2-E1 transition and the associated release of K^+ ions to the cytoplasm. Based on the results of stopped-flow kinetic experiments and recently published crystal structural data for the related enzyme, the sarcoplasmic reticulum Ca^{2+}-ATPase, it is suggested that the allosteric role of ATP in the mechanism of the Na^+,K^+-ATPase can be explained by an ATP-induced closing of the cytoplasmic domains of the enzyme which relieves steric hindrance arising from interactions between neighbouring pump molecules within the native membrane environment and hence an acceleration of the E2-E1 conformational change (Clarke, 2009). In the presence of millimolar concentrations of ATP, therefore, it is proposed that the enzyme functions as a monomer (alpha-beta protomer), whereas at low ATP concentrations it functions as a dimer ($(alpha\text{-}beta)_2$ diprotomer) or higher aggregate. The physiological advantage of protein-protein interactions is still unclear, but a possibility is that they may lead to an enhancement of the enzyme's ATP affinity and allow it to continue functioning even under hypoxic conditions.

Skou JC. (1957) *Biochimica et Biophysica Acta*, **23**: 394-401.
Clarke RJ (2009) *European Biophysics Journal*, in press.

3966-Plat
The Na/K-ATPase/Src Interaction and the E1/E2 Transition
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In our previous studies, we have shown that Na^+/K^+-ATPase interacts directly with Src to form a signaling receptor complex. This complex is involved in control of basal Src activity and ouabain-induced signal transduction. The aim of this work is to demonstrate that Na^+/K^+-ATPase also regulates Src activity through its conformation-dependent domain movements during pumping cycles. It is known that the Na^+/K^+-ATPase transits from E1 to E2 conformation during an ion pumping cycle. Based on the known crystal structures of Na/K-ATPase, this conformational transition results in a 110 degree turn of the A domain of Na/K-ATPase where Src SH2 domain binds. Computational modeling suggests that the movement of A domain during the pump cycle is likely to release Src from the Na/K-ATPase, resulting in the activation of Src kinase. Indeed, in vitro kinase assays show that while stabilization of Na/K-ATPase by N-ethylmaleimide and AMPPNP in E1P conformation keeps Src in an inactive state, converting the pump into an E2P state by fluoride compounds stimulates Src. Consistently, the Na^+-liganded E1 form of Na^+/K^+-ATPase inhibits Src whereas the K^+-liganded E2 form releases Src from the Na^+/K^+-ATPase and re-activated Src. Finally, Src is completely inactivated by the Na/K-ATPase in the presence of physiological concentrations of Na^+ (150 mM) and K^+ (5 mM). Reduction of K^+ results in an accumulation of E2P Na/K-ATPase and consequently an increase in active Src. Taken together, these new findings suggest that the Na^+/K^+-ATPase/Src complex may function as a pumping receptor, capable of coordinating ion pumping at the plasma membrane and other cellular activities by activating/inhibiting Src kinase.

3967-Plat
Oligomer Structure Detected in Na/K- and H/K-ATPase
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Since Repke's proposal of Flip-Flop model of Na/K-ATPase in 1973, Askari, Schoner, Hayashi, Taniguchi, Froehlich, Clarke and their coworkers have indicated the oligomericity of the enzyme from reactivity to various ligand (2006, J. Biochem. 140, 599 and also see review 2001, J.Biochem.129, 335). One of the most compelling pieces of evidence in favor of the oligomeric nature of Na/K-ATPase and gastric H/K-ATPase is the simultaneous presence of EP:EATP with half site phosphorylation and nearly half site ATP binding (1999, J. Biol. Chem.274,31792, 2002, Biochemistry 41, 2438). Recently