presence of these secondary mutations. Therefore, R1 and R4 both contribute to the positive electrostatic environment around E1. Moreover, $MTSET^+$ modification of E1C with R1E could only occur at hyperpolarizing voltages but not at depolarizing voltages, suggesting that R1 is proximal to E1 only at the resting state but moves distally at the activated state. Overall, our data is consistent with a mechanism where arginines interact sequentially with E1 as S4 moves from a resting to an activated conformation.

3234-Plat
A Single S4 Arginine is Sufficient for Voltage Sensitivity in the Hv1 Proton Channel
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Voltage-gated proton conductances (G_{vH+}) are found in a variety of cell types (e.g. alveolar epithelia and phagocytes) where they mediate an H^+-selective transmembrane ion efflux that alkalinizes the cell and provides charge compensation for NADPH oxidase. The voltage sensor domain (VSD) protein Hv1 is required for native G_{vH+} and sufficient to reconstitute the hallmark biophysical features of G_{vH+} in heterologous expression systems. Conserved Arg residues in the S4 transmembrane helix of VSD proteins are believed to constitute the primary voltage sensing elements. Voltage-dependent conformational rearrangements of S4 thus drive channel gating. The Hv1 contains three putative voltage-sensing arginines (R205, R208 and R211) in S4. In order to examine the contribution of S4 Arg residues to voltage-dependent activation in Hv1, we mutated each to Ala and measured expressed H^+ currents in voltage-clamped 293T cells. The effect of single mutations on the apparent threshold for voltage-dependent activation (V_{THR}) with symmetrical [H^+] ranged from negligible (R205A) to +77 mV (R211A). In order to determine the minimal number of S4 arginines that are required for channel opening, we constructed double mutations in S4 (R205A-R208A, R208A-R211A and R205A-R208A). Mutations bearing a single Arg in either position 208 or 211 generated measurable H^+ currents with dramatically shifted V_{THR} values (>+90 mV). A unique biophysical feature of G_{vH+} is the coupling of voltage and pH gradient sensing: voltage-dependent activation shifts ~40mV per pH unit change in the H^+ gradient ($\Delta pH = pH_{OUT} - pH_{IN}$). Interestingly, the slope of the $V_{THR}/\Delta pH$ relation was similar to *wt* Hv1 for all of the mutations tested. Our results demonstrate that a single S4 Arg is sufficient for voltage and ΔpH sensing in Hv1 and suggest that S4 arginines differentially contribute to the voltage sensing mechanism.

3235-Plat
Probing Energetic Contributions of Aromatic Residues at the Intracellular Gate of Shaker Potassium Channels
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Voltage-gated potassium channels contribute to cellular excitability by selectively gating the transmembrane passage of potassium ions. The intracellular bundle crossing of these channels is populated by aromatic phenylalanine and tyrosine residues that have been proposed to form a hydrophobic seal in the closed conformation of the channel. One such residue, Phe481, has resisted previous study by traditional site-directed mutagenesis because non-aromatic substitutions at this site fail to produce functional channels. We therefore expressed Shaker potassium channels carrying subtly altered phenylalanine residues with one, two or three added fluorine atoms to their aromatic ring, a manipulation which serves to serially reduce the negative electrostatic potential on the face of the aromatic while leaving the size and hydrophobicity of the side-chain virtually unperturbed. These unnatural phenylalanine derivatives were introduced at either of two positions, Phe481 and Phe484, near the bundle crossing with the in vivo nonsense suppression method to directly study the contribution of the electrostatic component of the side-chain to channel gating. In each case, the fluorinated phenylalanine side-chains were well tolerated, producing potassium channels with normal voltage-dependent activation and robust expression, albeit reduced when compared to wild-type channels. Serial fluorination at Phe481 lead to a stepwise left-shift of the conductance-voltage relationship (~6 mV for each added fluorine atom) and exponential fits showed channel deactivation slowed significantly at −60 mV, with time constants of ~3 ms and ~160 ms, for wt and the tri-fluorinated phenylalanine derivative, respectively. Conversely, serial fluorination at Phe484 had no effect on the voltage-dependence of activation or the time course of deactivation. Taken together, these results suggest that an electrostatic component of Phe481, but not Phe484, serves to stabilise the closed state of Shaker potassium channels.

3236-Plat
Strict Structural Requirements for Cholesterol to Inhibit BK Channels Point to Specific Steroid-Protein Interactions
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Cholesterol decreases large conductance, voltage/calcium-gated potassium (BK) channel activity (NPo), an action that solely requires the channel pore-forming (α) subunit and a minimum phospholipid environment (Crowley et al., 2003). This cholesterol action is attributed to cholesterol-induced tight packing of bilayer lipids (Chang et al., 1995). Cholesterol modulation of ion channels *via* direct protein-steroid interactions, however, is increasingly recognized (Epshtein et al., 2009). Cholesterol analogs have been widely used to distinguish between lipid bilayer-mediated and specific protein recognition mechanisms. Thus, we probed cholesterol analogs on BK α subunits cloned from rat cerebral artery myocytes ("cbv1"; AY330293) after channel reconstitution into 3:1 (w/w) POPE:POPS bilayers. Cholesterol (33 mol%) decreased cbv1 NPo by ≈25%. In contrast, 5-cholenic acid-3β-ol, having a carboxyl group at the lateral chain end, failed to decrease NPo, underscoring the importance of a hydrophobic chain for sterol insertion into the bilayer hydrophobic core and channel inhibition. Coprostanol and cholestanol having the A/B junction in *cis* and *trans,* respectively, also decreased NPo (≤25%). In contrast, cholesterol, coprostanol and cholestanol epimers, having the C3-hydroxyl group in α-configuration, failed to decrease NPo. Therefore, a β-conformation in the hydroxyl is necessary for these monohydroxy-sterols to inhibit BK channels, strongly suggesting specific, steroid-protein interactions. Moreover, we probed the cbv1 channel with enantiomeric cholesterol (*ent*-cholesterol), which has physico-chemical properties similar to those of cholesterol yet can be differentially sensed by protein sites, as demonstrated by the lack of viability of *C. Elegans* when only *ent*-cholesterol is present (Crowder et al., 2001). Remarkably, *ent*-cholesterol repeatedly failed to reduce cbv1 NPo, buttressing the idea that cholesterol inhibition of BK channels requires steroid recognition by protein site(s), likely present in the cbv1 subunit itself.
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Platform BA: Emerging Single Molecule Techniques II

3237-Plat
High-Resolution smFRET in a Microfluidic Gas Exchange Platform
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Single-molecule Förster Resonance Energy Transfer (smFRET) is a powerful technique providing new insights in the physics and chemistry of bio-molecules. The smFRET signal quality depends on the photon-flux that can be harvested from the fluorescent dyes. Mere use of high excitation intensities produces counter-productive results, mainly because oxygen-mediated photobleaching of the fluorophores rapidly destroys useful FRET signals. Here, we developed a microfluidic device that ensures a large decrease in the oxygen content of buffers in-situ, allowing us to substantially reduce photobleaching even at high laser powers and obtain dramatic enhancement of signal for several dye-pairs. The principle of the deoxygenation is simple: the microchannels carrying the samples are flanked by large channels ventilated by nitrogen; oxygen is removed by molecular diffusion through porous walls. The device combines this deoxygenation with on-chip mixing and generation of dual-component triplet quenchers (increasing the burst brightness), and also has laminar-flow mixing for kinetic studies.
The increased photon flux obtained in the flow device leads to resolution improvements in two key dimensions: (i) it reduces considerably the time (by a factor of ~ 10 from the typical 500μs) needed to collect high-quality FRET signal, thus providing higher time-resolution, and (ii) it allows the use of higher thresholds, which significantly reduces the width of the smFRET distributions and allows a better resolution of molecular subpopulations. In contrast with a popular enzymatic oxygen scavenger, the method can be used for de-oxygenation in denaturing conditions, hence enabling studies of protein folding. Overall, the platform combines multiple enabling features that can accommodate a range of equilibrium and kinetic and biochemical experiments, along with

a simple construction and fabrication, and robust operation. Together, these features have the potential to make it useful for a broad variety of single-molecule fluorescence experiments.

3238-Plat
Single Plane Illumination Microscopy Allows Fluorescence Correlation Spectroscopy (SPIM-FCS) to be used for Concentration and Diffusion Coefficient Imaging in 3d
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Fluorescence Correlation Spectroscopy (FCS) is a widely used tool for the investigation of molecular dynamics in vitro and in vivo. However, it is mostly used in a confocal configuration, which renders multiplexing difficult. Recently, camera based approaches have been used to measure a large number of correlation functions in parallel. Here, we introduce single plane illumination microscopy (SPIM) as an illumination mechanism for FCS. An illumination objective creates a light sheet in the focal plane of a detection objective, which excites a volume that is comparable to the focal volume of the detection system. The image is collected with a fast EMCCD camera. The signal from every pixel is analysed by calculating a correlation function thus extracting concentrations and diffusion coefficients. Apart from the fact that SPIM illuminates only that fraction of a sample, which is actually measured and thus provides true optical sectioning, it reduces photobleaching compared to confocal setups limiting optical damage to the sample and allowing longer measurement times. We use a laser power in front of the objective of 60 μW and record at least a 32×32 pixel area of an EMCCD. This corresponds to a reduction in laser power delivered to the specimen by at least 3 orders of magnitude compared to confocal FCS. In this work, we demonstrate the use of different combinations of low numerical aperture (NA) illumination objectives for the creation of light sheets with high NA detection objectives for SPIM-FCS. By using microsphere solutions we were able to distinguish the diffusion coefficient of different sized particles and demonstrated that FCS images with 1024 pixels up to 4096 pixels show a clear contrast in concentration and diffusion coefficients in non-homogeneous samples.

3239-Plat
High Throughput Single-Molecule Spectroscopy with Highly Parallel Excitation and Detection
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Single-molecule spectroscopy is a set of powerful techniques for detailed analysis of molecular interactions and motion, but due to the requirement of low concentrations, long acquisition times are required to achieve sufficient statistics. Newly developed detectors permit acquiring data in parallel, resulting in faster acquisition and giving access to shorter time scales for the observation of dynamic phenomena. We present several new single-photon counting detectors and acquisition systems capable of high throughput single-molecule spectroscopy including multi-pixel CMOS detectors and a multi-pixel hybrid photodetector. To take advantage of these detectors, we developed a novel approach for multi-spot excitation utilizing a liquid crystal on silicon spatial light modulator (LCOS), which allows dynamic excitation spot generation corresponding to the detector geometry. We also developed a high throughput field programmable gate array (FPGA)-based parallel acquisition system. We present examples of these approaches for single-molecule applications such as Fluorescence Correlation Spectroscopy (FCS), and demonstrate the feasibility for high throughput single-molecule Förster resonance energy transfer (smFRET) measurements. We also discuss the ways in which our approaches permit measurements of faster dynamic processes. These high throughput developments will significantly expand the power of single-molecule spectroscopy for biophysical and other applications.

3240-Plat
Measuring Multiple Distances within a Single Molecule using Switchable FRET
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Single molecule Förster Resonance Energy Transfer (smFRET) is a powerful method that serves as a nanometer-scale ruler to probe structure and conformational dynamics of biomolecules. In most cases, smFRET measurements utilize a single donor-acceptor pair reporting on a single distance. The ability to monitor FRET between multiple donor-acceptor pairs within a single molecule (multi-pair smFRET) will provide new fundamental insight into the three-dimensional structure, dynamics and interactions of biomolecules and benefit many fields such as structural biology and biosensing. Although multi-pair smFRET approaches have been reported, they are complicated, non-general, and difficult to extend.
Here, we present a novel, flexible, and general multi-pair smFRET method that uses photoswitchable fluorophores as FRET-acceptors. It allows the measurement of multiple distances on an individual molecule such as a protein, protein-DNA complex, or multi-protein complex. Our proof-of-principle experiments were performed on double-stranded DNA fragments labeled with a non-switchable donor and multiple photoswitchable acceptors. Using alternating-laser excitation schemes and stochastic photoswitching of acceptors, we demonstrate the measurement of multiple distances within an individual DNA molecule (as opposed to within a population of single molecules). Our results are complemented and supported by simulations of the photoswitching process and the associated single-molecule fluorescence observables. Moreover, by employing a step-finding algorithm, we achieve high-resolution FRET measurements within an individual molecule. Finally, we show that different mechanisms for photoswitching can be used to the same end, supporting the generality of the concept.

3241-Plat
Colloidal Lenses Enable High Temperature Single Molecule Imaging and Improve Fluorophore Photostability
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Although single molecule fluorescence spectroscopy was first demonstrated at near-absolute zero temperatures (1.8 K), the field has since advanced to include room temperature observations largely due to the use of high numerical aperture objective lenses, brighter fluorophores, and more sensitive detectors. This has opened the door for many chemical and biological systems to be studied at native temperatures at the single molecule level both in vitro and in vivo. However, systems and phenomena that operate at temperatures above 37 °C remain difficult to study at the single molecule level due to the need for index matching fluids with high numerical aperture (NA) objective lenses. These fluids act as a thermal conductor between the sample and the objective and sustained exposure to high temperature can cause the objective to fail. This has prevented the single molecule study of thermophilic organisms, the interactions of their protein repertoire, and the temperature-dependent unfolding kinetics of nucleic acids and proteins. Here we report that high index of refraction micron-sized colloidal lenses are capable of achieving single molecule imaging at 70 °C by incorporating a focusing element in immediate proximity to an emitting molecule; the optical system is completed by a low numerical aperture optic which can have a long working distance and an air interface. TiO2 colloidal lenses were used for parallel imaging of surface-immobilized single fluorophores and to measure real-time single molecule mesophilic and thermophilic DNA polymerase strand displacement replication through an immobilized template at 23 °C and 70 °C, respectively. Fluorophores in close proximity to TiO2 also exhibited a ~40% increase in photostability due to a reduction of the excited-state lifetime.

3242-Plat
Synthesis of Extended Single-Molecule Optical Encoders
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We have designed single-molecule FRET encoders which convert relative motion between two individual biomolecules to a periodic signal. Fundamental signal frequencies obtained with individual DnaB helicase molecules imply unwinding velocities ranging from 200 to 1100 base pairs per second, while low-frequency modulation of peak heights may suggest azimuthal rotation of the helicase. Signal durations show that a single helicase is capable of unwinding many hundreds of base pairs before dissociating. The initial scheme chosen for FRET encoder synthesis was expensive and limited to 5 periods, restricting the duration of the collected signal. We have utilized polymerization-driven self-assembly and rolling circle replication to synthesize inexpensive, extended FRET encoders as long as 150 periods.