Short double-stranded RNA molecules have recently emerged as important regulators of gene expression. These small RNAs associate with a member of the Argonaute protein family in an assembly known as RNA-induced silencing complex (RISC).
Here we elucidate the pathway of RNA Interference (RNAi) in vivo by applying fluorescence correlation and cross-correlation spectroscopy (FCS/FCCS). We show that two distinct RISC exist: a large ~3 MDa complex in the cytoplasm and a 20-fold smaller complex in the nucleus. Nuclear RISC, consisting only of Ago2 and a short RNA, is loaded in the cytoplasm and imported into the nucleus. The import of Ago2 into the nucleus is mediated by the import receptor Impotin8.
We further demonstrate that FCCS can be used to study the interaction of different members of the Argonaute protein family with short double-stranded RNAs and their target mRNA molecules.

2136-Plat
G-Quadruplex Folding Observed by two Photon Fluorescence Correlation Spectroscopy and Dual Time Scale
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G-rich DNA sequences are known to fold upon addition of salt into a stacked well defined configuration called a quadruplex. A fluorescently labeled 5′-FAM −24-mer G-quadruplex sequence was used to explore the variation of diffusion coefficients at extremely low, low and high KCl concentrations. We found a shift in the diffusion coefficient of about 10μm2/sec toward faster diffusion from extremely low to high KCl concentrations. This shift can be related to the compact structure formed by the G-quadruplex. We have also used a fluorescent guanosine analog, 6MI, to label a 24mer that has shown folding behavior at high KCl concentrations. To explore this further, we have added in excess a sequence that complements the G-rich region to deter the formation of the G-quadruplex. The diffusion coefficient also increased from the unfolded, low KCl concentration to the high salt, G-quadruplex structure. We have constructed a dual-timescale (ps TCSPC and uS-mS FCS) photon correlation system and we are using it to explore linked changes in the fluorophores' lifetimes and the translational diffusion coefficients as they move between low and high salt environments. Part of this work was supported by NIH SCORE Grant S06 GM 060654.

2137-Plat
Observing Nuclear Receptor / Coactivator Interactions in Live Cells by Hetero-Species Partition Analysis
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Measuring the binding curve and stoichiometry of protein complexes in living cells is a prerequisite for quantitative modeling of cellular processes. Dual-color fluorescence fluctuation spectroscopy provides a general framework for detecting protein interactions. However, quantitative characterization of protein hetero-interactions remains a difficult task. To address this challenge we introduce hetero-species partition (HSP) analysis for measuring protein hetero-interactions of the type D + nA -> DA_n. HSP directly identifies the hetero-interacting species from the sample mixture and determines the binding curve and stoichiometry in the cellular environment. The method is applied to measure the ligand-dependent binding curve of the nuclear receptor retinoic X receptor to the coactivator transcription intermediate factor 2. The binding stoichiometry of this protein system has not been directly measured yet. A previous study using protein fragments observed a higher binding stoichiometry than biologically expected. We address this difference in stoichiometry by measuring the binding curves of the full-length proteins in living cells. This study provides proof-of-principle experiments that illustrate the potential of HSP as a general and robust analysis tool for the quantitative characterization of protein hetero-interactions in living cells.

Platform AE: Muscle Regulation

2138-Plat
Determining Mechanism of Phosphorylation of Smooth Muscle Myosin by Calmodulin-Myosin Light Chain Kinase Using an in vitro Model System
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We have shown that MLCK and calmodulin (CaM) co-purify with unphosphorylated SMM (up-SMM) from chicken gizzard, suggesting that they are tightly bound. Although the MLCK:SMM molar ratio in SMM preparations was well below stoichiometric (1:73 ± 9), the ratio was ~ 23-37% of that in gizzard tissue. Fifteen to 30% of MLCK was associated with CaM at ~1 nM free [Ca^{2+}]. There were two MLCK pools that bound up-SMM with Kd ~10 μM and 0.2 μM and phosphorylated SMM with a Kd ~ 20 μM and 0.2 μM. Using motility assays, co-sedimentation assays, and on-coverslip ELISA assays, we provide strong evidence that most of the MLCK is bound directly to SMM through the telokin domain. The bound MLCK can phosphorylate SMM in a Ca^{2+}-dependent manner with a pCa_{50} ~ 6 as measured by in vitro motility, similar to in vivo results. After activation of SMM-bound MLCK/CaM with Ca^{2+} and ATP, both motility (0.5 μm/sec) and phosphorylation (>15%) of SMM reach a maximum after ~15-30 min, inconsistent with a free diffusion mechanism. Actin movement over the SMM is not required for this phosphorylation process. Experiments are underway to test the idea that SMM heads proximal to the MLCK-SMM become phosphorylated by a tethered diffusion mechanism.

2139-Plat
The Crystal Structure of the N-terminal 15 Heptads of Smooth Muscle Myosin Rod Offers Insights into the Inhibited State of Myosin
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The coiled coil rod of smooth muscle myosin is important both for regulation of activity and optimal mechanical performance. Myosin with a phosphorylated light chain is active, while in the inhibited, dephosphorylated state the two heads form an asymmetric intramolecular interaction. The minimal myosin that can attain an ''off'' state has two heads and 15 heptads of coiled coil rod, a length approximately equal to that of the myosin head. This observation implies that there may be head-rod interactions in the inhibited state. Here we have determined the crystal structure of this region of the rod. Despite being a parallel, coiled coil dimer, the core arrangement is asymmetric. We propose that this asymmetry is wired into its sequence and crucial to its function. The core of the S2 segment is loosely packed in stretches and the two helical segments are locally off-register or staggered relative to one another. Staggered regions are centered on non-canonical core residues. This relative staggering causes three prominent bends in the coiled coil. Significant deviations from two-fold symmetry are observed in our structure, and to a lesser extent in equivalent crystal structures of S2 fragments from cardiac myosin. The larger variations in stagger and bend angles in the rods of smooth versus striated muscle myosins may explain in part why asymmetric head-head interactions are more prevalent in the thick filament regulated myosins.

2140-Plat
Electron Microscopy and Molecular Dynamics on a D137L Mutant of Tropomyosin
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It is generally agreed that constraints on the curvature and flexibility of tropomyosin are necessary both for the binding and regulatory movements of tropomyosin on actin filaments. It follows that mutagenesis of residues that may affect curvature and/or flexibility is commonly used as an analytical tool. The tropomyosin coiled-coil is stabilized by hydrophobic residues in the "a" and "d" positions of its heptad repeat. However, a highly conserved Asp137 places a negative charge on each chain in a position typically occupied by hydrophobic residues. Substituting a canonical Leu for Asp137 suggested that Asp137 destabilizes tropomyosin and imparts flexibility (Sumida et al., 2008). The D137L mutant does retain F-actin binding properties. We have now assessed changes of curvature and flexibility by EM and Molecular Dynamics (MD) on the Leu137 mutant. Contrary to expectation, rotary shadowed D137L tropomyosin is more curved, not straighter, than control tropomyosin. Moreover, overall the average MD shape of the molecule is extremely bent and, unlike wild type tropomyosin, does not match the contours of the F-actin helix at all. We find that the persistence length of D137L is half that of wild-type tropomyosin (measured either on EM images or on MD frames), indicating that the mutant is more curved and more flexible than the wild type is. MD shows that there is a modest decrease in curvature in the surrounds of residue 137 in the D137L mutant, but it is accompanied by a large unexpected increase in curvature near residue 175. Thus we find that mutation at one site on tropomyosin leads to an unexpected delocalized change at another site along the molecule.