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.

2141-Plat

Troponin Regulatory Function and Dynamics Revealed by H/D Exchange-Mass Spectrometry

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Troponin is the thin filament protein that confers tight, Ca^{2+}-dependent control over muscle contraction. The mechanism of this regulation was investigated by detailed mapping of the dynamic properties of cardiac troponin, using amide hydrogen exchange-mass spectrometry, in the presence of either saturation or non-saturation of the regulatory Ca^{2+} binding site in the NH_2-domain of subunit TnC. Troponin was found to be highly dynamic, with 60% of amides exchanging H for D within seconds of exposure to D_2O. In contrast, portions of the TnT-TnI coiled-coil exhibited high protection from exchange, more than six hours, identifying the most stable portion of the trimeric troponin complex. Regulatory site Ca^{2+} binding altered dynamic properties (i.e., H/D exchange protection) locally, near the binding site and in the TnI switch helix that attaches to the Ca^{2+}-saturated TnC NH_2-domain. More notably, Ca^{2+} also altered the dynamic properties of other parts of troponin: the TnI inhibitory peptide region that binds to actin, the TnT-TnI coiled-coil, and the TnC COOH-domain that contains the regulatory Ca^{2+} sites in many invertebrate as opposed to vertebrate troponins. Mapping of these affected regions onto troponin's highly extended structure indicates contacts important in conformational change: in the low Ca^{2+} state the TnI region that effects inhibition bends back and interacts with the end of the TnT-TnI coiled-coil, as previously suggested by intermediate resolution X-ray data of skeletal muscle troponin. Thus, troponin-mediated Ca^{2+} sensitive regulation of muscle contraction consists of Ca^{2+}-triggered switching between alternative sets of intra-troponin interactions.

2142-Plat

Magnesium Stabilizes the Closed Conformation of the C-Domain of Troponin C

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Activation of the thin filament in striated muscles requires both the binding of Ca^{2+} to the N-domain of troponin C (TnC) and the binding of myosin cross-bridges to actin, which has been shown to alter the C-domain conformation. Here we have evaluated the structural and functional consequences of divalent cation exchange in skeletal and cardiac TnC (sTnC and cTnC). We have used intrinsic tyrosine fluorescence, circular dichrosim (CD), and the fluorescent nonspecific hydrophobic probe bis-ANS to monitor changes in domain conformation in response to Ca^{2+} and Mg^{2+} binding in the sTnC, cTnC, and in a cTnC mutant in which the invariant Glu residue at the 12th position of the calcium binding loops III and IV were substituted with Asp (cTnCDD). Ca^{2+} binding causes an increase in Tyr fluorescence and α-helical content in sTnC and cTnC, but not in cTnCDD. Ca^{2+} induced C-domain opening characteristic of sTnC and cTnC was also greatly reduced in cTnCDD, as measured by bis-ANS fluorescence. Thus the Asp to Glu substitutions appear to prevent the C-domain from opening. Bis-ANS Ca^{2+} titrations also showed that high Ca^{2+} concentrations may be sufficient to open the N-domain of cTnC, which was reported to remain in the closed conformation in the Ca^{2+}-bound state. Lastly, bis-ANS Mg^{2+} titrations indicate that Mg^{2+} does not cause domain opening in either cTnC or cTnCDD. The closed conformation of the Mg^{2+}-bound C-domain of TnC implies a different mechanism of interaction with TnI than that in the presence of Ca^{2+} and suggests that the Mg^{2+}-Ca^{2+}exchange in TnC may contribute to the thin filament activation of muscle contraction. This conclusion is consistent with our observation that physiological concentrations of Mg^{2+} significantly lower the Ca^{2+}-sensitivity of reconstituted cardiac thin filaments.

2143-Plat

Phosphomimetic Substitutions in One or Both Ser43/45 Residues of Cardiac Troponin I Produces Comparable Changes in Contractile Performance

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Cardiac troponin I (cTnI) is phosphorylated on three clusters of residues in response to protein kinase C (PKC) activation. Previously, studies on the cTnISer43/45 cluster showed phosphomimetic Asp substitution reduced peak shortening and accelerated re-lengthening in adult cardiac myocytes. The goal of the present study is to determine whether one or both Ser residues contribute to the functional response observed with cTnISer43/45Asp. We studied adult rat cardiac myocytes 2 and 4 days after viral-mediated gene transfer of cTnIFLAG, cTnISer43Asp or cTnISer45Asp (+FLAG). Western analysis indicated similar levels of cTnI replacement developed for all groups, and extensive replacement with cTnIFLAG ($71 \pm 9\%$, n= 6), and FLAG-tagged epitopes of cTnIS43D ($72 \pm 3\%$, n=8) and cTnIS45D ($70 \pm 5\%$, n=8) within 4 days. Further analysis showed no significant change in cTnI stoichiometry and confocal analysis confirmed a sarcomeric incorporation pattern for each mutant. In functional studies, shortening amplitude decreased significantly in chronically paced myocytes expressing non-tagged Ser43Asp and/or Ser45Asp compared to controls (Control = 0.149 ± 0.008 μm, n=36; cTnISer43/45Asp = 0.110 ± 0.006 μm; n=32*; cTnISer43Asp = 0.095 ± 0.007, n=44*; cTnISer45-Asp = 0.108 ± 0.007*, n=50; *$p<0.05$ vs control) 4 days after gene transfer. An accelerated re-lengthening accompanied this reduced shortening (Time to 75% relaxation = $TTR_{75\%}$ (ms): Control = 79 ± 4; cTnISer43/45Asp = 62 ± 4*; cTnISer43Asp = 63 ± 4*, cTnISer45Asp = 65 ± 3*; *$p<0.05$ vs control). Interestingly, each single mutant also accelerated the time to peak shortening (TTP (ms): Control = 83 ± 5; cTnISer43Asp = 68 ± 3*; cTnISer45Asp = 67 ± 2*; *$p<0.05$ vs control) while cTnISer43/45Asp did not (84 ± 5). These initial results provide evidence that each Ser residue in the Ser43/45 cluster is capable of altering cTnI function in response to phosphorylation by PKC, yet phosphorylation of both residues does not produce an additive response.

2144-Plat

Effect of Troponin Ca^{2+} Binding Properties on the Kinetics of Myofibril Force Initiation and Relaxation

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We have engineered the Ca^{2+} binding properties of troponin C (TnC) to study the role of increased (I60Q sTnC) and decreased (M80Q $sTnC^{F27W}$) Ca^{2+} dissociation rate (k_{off}) on activation and relaxation of skeletal muscle. Previously we reported that myofibril force development kinetics (k_{ACT}) are not influenced by decreasing k_{off} from Tn, but are slowed by an increase in k_{off} (Kreutziger et al. 2008 JPhsiol. 586;3683-3700) at low [P_i] (5 μm). The time to initiation of force ($k_A$lag) following a rapid (~10ms) switch from pCa 9.0 to pCa 3.5 provides information about thin filament activation rate and our preliminary data suggest this rate may also be sensitive to k_{off}. In rabbit psoas myofibrils (15°C) $k_A$lag (~20 ms for native or WT sTnC) is almost eliminated for M80Q $sTnC^{F27W}$ and increased by I60Q sTnC (~40-50 ms). Additionally, though k_{ACT} is similar for force increases from either full or partial activation to full activation, $k_A$lag disappears when starting from partial activation. We have also reported that fast and slow phase rates of relaxation are not affected by k_{off}, but that duration of the slow phase is affected in skeletal myofibrils. Here we report that lag prior to initiation of the slow phase ($k_R$lag) may be also influenced by k_{off}. Opposite to $k_A$lag, $k_R$lag (~20 ms for WT or native sTnC) was increased (~40-50 ms) by decreased k_{off} (M80Q $sTnC^{F27W}$) and almost eliminated by increased k_{off} (I60QsTnC). These experiments demonstrate a potential approach to study thin filament activation/deactivation kinetics without the need for fluorescent probes attached to thin filament proteins that can affect their function. Supported by Telethon GGP07133, MIUR (CP, CT), NIH-HL65497 (MR).

2145-Plat

Nebulin Alters Crossbridge Cycling Kineticis and Increases Thin Filament Activation - a Novel Mechanism for Increasing Tension and Reducing Tension Cost

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Nebulin is a giant filamentous F-actin binding protein that binds along the thin filament of the skeletal muscle sarcomere. Although nebulin is usually viewed as a structural protein, here we investigated whether nebulin plays a role in muscle contraction by using skinned muscle fiber bundles from a nebulin knockout (NEB KO) mouse model. We measured force-pCa and force-ATPase relations, as well as the rate of tension redevelopment (k_{tr}) in tibialis cranialis fibers. To rule out any alterations in troponin (Tn) isoform expression and/or status of Tn phosphorylation, we studied fibers that had been reconstituted with fast skeletal muscle recombinant Tn. We also performed a detailed analysis of myosin heavy chain, myosin light chain (MLC) and MLC2