endogenous sTnC and reconstituting with I60Q sTnC in demembranated rabbit psoas fibers (n=8) caused a decrease in maximal force (F_{max}) to 65±3.4% of WT and a dramatic loss of Ca^{2+} sensitivity (pCa_{50}) of 1.15 pCa units from 5.74±0.05 to 4.59±0.05 at 15°C with 2.5μm sarcomere length. The slope of the force-pCa curve decreased from 2.1±0.1 to 1.7±0.1. At maximal activation (pCa 4.0), phosphate (P_i) sensitivity of force and the rate of force redevelopment (k_{tr}) significantly increased with I60Q sTnC. The reduced F_{max}, pCa_{50} and slope and increased Pi sensitivity is similar to our previous measurements where thin filaments were reconstituted with a mixture of 20% purified sTnC and 80% D28A, D63A sTnC (no Ca^{2+} binding at the N-terminus). This 20% to 80% mixture reduced nearest neighbor regulatory unit (comprising 1 troponin, 1 tropomyosin, and 7 actins) coupling by spatial means. We conclude I60Q sTnC compromises spread of activation along the thin filament though kinetic means, even though the spatial relationship between neighboring regulatory units is maintained. Our results support the hypothesis that coupling between thin filament regulatory units dominates cooperative thin filament activation, and suggests it may limit force development kinetics when creatine phosphate levels decrease and phosphate levels increase (as in fatigue).

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960-Plat Structural and Functional Implications of Familial Hypertrophic Cardiomyopathy Mutations in cTnT

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Familial Hypertrophic Cardiomyopathy (FHC), a primary disease of the cardiac sarcomere, is a common cause of sudden cardiac death in young people. Many disease-causing mutations in the thin filament protein cTnT are found in TNT1, a tropomyosin binding region. Residues 160–163, which fall within a highly charged region (RREEEENRR), represent a mutational hotspot where the α-helix may unwind to provide flexibility necessary for function. The flexibility of this putative hinge region may be affected differently by mutation of these residues, and these differences may provide insight into the structure of TNT1 and the molecular mechanism of FHC. Our SDSL-EPR data suggest that the C-terminal portion of this region has loop-like characteristics in the troponin complex. In order to study the effects of these mutations, cTnT residues 120–220 were modeled *in silico* and the molecular dynamics of the mutations were compared to wild type. Interestingly, preliminary MD simulations indicate that deletion of residues 160E and 163E may have the greatest effect on the flexibility of this region, but with opposite effects. Δ160E decreases simulated flexibility, while Δ163E increases flexibility. The positions of these mutations in the hinge region may affect troponin complex assembly, consequently altering protein flexibility and impairing function. Residue 160E may be a part of a helical region of the protein, while 163E is located in a less structured portion, accounting for the different effects predicted for these mutations. To evaluate our MD studies *in vivo*, a transgenic mouse model of Δ163E has been generated, and the physiologic effects will be compared to the existing murine Δ160E model. Additionally, further EPR studies will be performed on WT, Δ160E, and Δ163E to elucidate the structure about this hinge region and the effects of these mutations on troponin complex structure.

Platform Z: Fluorescence Spectroscopy II

961-Plat Study Of Nuclear Receptor-Coregulator Interactions In Live Cells By Two Photon Two Colour Fluorescence Cross Correlation Spectroscopy

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Nuclear receptors (NR) play key roles in a multiple cellular functions and are implicated in a large number of human pathologies. As such they represent major targets of drug development programs, and many of their ligands are used therapeutically. Quantitative information about their ligand-dependent interactions in live cells would greatly enhance our understanding of their function and our ability to modulate their activity.

The goal of this research is to quantitatively characterize, in live cells, the interactions between nuclear receptors (NR) and their coregulator partners with the aim of more clearly defining their functional mechanisms. We use two photon two colour fluorescence cross correlation spectroscopy (TPTCFCCS). This approach allows for combining 2-photon 3D microscopy with correlation spectroscopy. By using interacting protein pairs expressed as fusions of two different colored fluorescent proteins, it is possible to measure quantitatively the degree of interaction between the NR and their coregulator partners in live cells. Our study focuses on the NR implicated in estrogen signalling, homodimers of human estrogen receptors (ER) α and β, and their major transcriptional regulatory partners, in particular TIF2 and RIP140, in Cos-7 cells. We first constructed cerulean-ERα, cerulean-ERβ, cherry-RIP140 and cherry-TIF2, and tested their activity in transiently transfected cells in the presence or absence of agonist (estradiol, E2) or antagonist (ICI). We next analyzed quantitatively their interactions by TPTCFCCS. We also investigated the formation of ERα/ERβ heterodimers.

The presence of agonist leads to nearly 100% interaction between ERα-TIF2 and ERβ-TIF2, while antagonist totally inhibits the formation of these complexes. Similar results were obtained for ER-RIP140 interactions.

962-Plat Molecular Thermometers For Temperature Measurements In Biological Systems

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Due to the miniaturization of biological and chemical systems, new approaches are needed to measure physiological parameters such as pH and temperature in tiny fluid samples such as living cells. We describe a noninvasive, spatially resolved method to determine the absolute temperature of small volumes of fluid using the enhanced green fluorescent protein (EGFP) as a molecular indicator. Our approach is based on the detection of the strongly temperature dependent relaxation time associated with the fluorescence blinking of the protein using fluorescence correlation spectroscopy (FCS). We applied this approach to measure the temperature change due to laser heating effect of a thin liquid sample. Our results showed that there was a linear dependence between the temperature change at the laser focus and both the laser power and the extinction coefficient of the sample as expected. Furthermore, we were able to measure the laser induced temperature change at the liquid/glass interface of the sample. We also investigated the use of pyranine as a molecular thermometer. This fluorophore is known to have a pH dependent reversible protonation reaction affecting its fluorescence. We observed that the relaxation time and the equilibrium state associated with blinking are dependent on pH, temperature, and buffer concentration. We showed that we can determine both the pH and the temperature of a sample by measuring these two parameters. Although EGFP's blinking has a stronger temperature dependency at low pH, pyranine is more useful for measurements in physiological conditions due to its higher pKa value.

963-Plat Rotation and Reaction of Biomolecules Confined to Sub-Femtoliter Volumes

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We measure the rotational and chemical dynamics of molecules confined to sub-femtoliter aqueous droplets that can be optically trapped ("hydrosomes'). In small volumes, as in the confining environment of cells and organelles, it is necessary to ask what the effect of the boundaries and confinement are on the physical and chemical dynamics of the contained molecules. We begin to answer this question by measuring the rotational dynamics of confined molecules, including proteins, DNA and RNA. Time resolved fluorescence anisotropy is used as a diagnostic for tumbling rates of molecules. For most water-soluble molecules investigated, we find the tumbling rates inside the hydrosome are similar to those found in bulk solution. However, we do find that surfactants play a crucial role in maintaining the integrity of confined biomolecules, in particular for proteins such as EGFP. To study biochemical reactions in the confining environment of hydrosome, we have developed and report on an automated dual micro-injector based platform for generating, mixing, and interrogating the contents of hydrosomes. Two sub-femtoliter aqueous droplets, which contain biomolecules of different reagents, can be generated on demand from a dual-injector apparatus and then fused, which facilitates assembly of two separate moities to form a molecular complex. In a typical experiment, hydrosomes are injected and then, by computerized control of a piezoelectric translation stage and single particle video tracking techniques, automatically trapped and mixed by dual optical tweezers to trigger a reaction process with precise timing. We demonstrate application of this droplet-mixing technique to study the reactions of calcium/fluo-3 binding and protein/DNA interactions.

964-Plat Fluorescence Microscopy in a Microwave Cavity

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Prolonged exposure to microwave irradiation has been considered deleterious to biological systems and biomolecules. Subsequently, the effect of microwave exposure on biological systems has drawn considerable interest with regards to the hazards of prolonged exposure to microwave irradiation. With regard to these studies (1), most of these works describe the before and after effects of microwaves on biological systems with a few exceptions (2–4). Recently, (Copty, et al, 2007) the microwave effects on green fluorescent protein (GFP) were studied in real-time to highlight one of only a very few examples. In this work, we subsequently demonstrate the development of a fluorescence microscope in a microwave cavity to image microwave effects on temperature dependent solutions, in real-time. We believe that this real-time imaging system could greatly facilitate the understanding of microwave effects on enzyme reactions rates, biomolecular interactions, and living biological organisms, i.e. mammalian cells. With this technology, we provide a methodology to potentially record real-time single molecule data in microwave cavities during the application of a microwave field using techniques, such as FRET and FCS.

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965-Plat Estimate of the FRET Dipole Orientation Factor from the Polarization Anisotropy of Fluorescence Signal

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The measurement of Förster resonance energy transfer (FRET) efficiency between donor and acceptor fluorophores attached to the

same complex can be used to estimate the distance between fluorophores. FRET efficiency depends not only on the distance, but also on the orientation of the fluorophore dipoles, described by the dipole orientation factor κ^2, which can take values from 0 to 4. Although it is impossible to measure distributions of all three angles contributing into κ^2, under certain assumptions we can restrict the admissible range of κ^2 using fluorescence emission anisotropy (EA) r of the donor alone, the acceptor alone, and the FRET signal. The method was derived in 1979 [1], but more recently the common practice appears to have been using the time-average value of the dipole orientation factor 2/3, which assumes loss of the acceptor dipole orientation during the donor fluorescence lifetime. The reported values of the donor and acceptor EA are frequently close to 0.2 or even higher, which leads to the significant uncertainty in the distance measurement (about +15% −20% or more). The equation for the dipole orientation factor derived in [1] can be rewritten in a simpler form as the direct function of the EAs and updated with additional geometric constraint for remaining unknown angles (inequity, missing in the paper [1]). The extrema search in the paper [1] was performed incorrectly, and the plots are complicated, but all the numerical calculations can now be easily performed using computer.

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966-Plat 4Pi Microscopy for Fluorescence Microphotolysis and Correlation Spectroscopy

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Light microscopy remains currently the main tool for studying living cells, but its resolution has been limited to the micrometer scale due to the nature of light diffraction. This limit has been overcome recently by a new generation of light microscopes, such as the 4Pi microscope which offers a resolution of about 100 nm along its optical axis. However, a theoretical-computational framework is needed to employ the new instruments in such applications as continuous fluorescence microphotolysis (CFM) and fluorescence correlation spectroscopy (FCS). We developed such a framework and validated it through measurements on model systems, employing 4Pi as well as confocal conditions together with either single- or two-photon excitation. In all cases experimental data can be well matched to computed signals, yielding diffusion coefficients of the fluorophores used. We also analyzed optical setups similar to that of the 4Pi microscope, but focusing large numbers of interfering laser beams. We find that the instrument point spread function in such case can be substantially narrowed beyond that of the 4Pi, i.e., two-beam, case, but that narrowing reaches quickly a finite limit, the largest narrowing arising in going from one to two to three beams.

967-Plat Dynamic Stokes Shift and Excited-State Trp Population Decay in E21W and K58W mutants of IIA-Glc protein in H2O and D2O

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Escherichia Coli IIA^{Glc} is a stable globular protein containing neither tryptophan nor tyrosine. To study the effect of nearby charges on tryptophan fluorescence we made the E21W and K58W variants of IIA^{Glc}. The crystal structure of E21W IIA^{Glc} reveals that the backbone conformation is unchanged from wild-type. The distance between well-ordered W21 and positively charged R165 is less than 4Å. Model building experiments suggest that in K58W, the distance between W58 and negatively charged E121 does not exceed 6Å.

Time-resolved fluorescence emission spectra of the E21W and K21W variants were measured in H_2O (pH=7.50) and in D_2O (pD=7.50). No changes in the shapes or the linewidths of the spectra were found, which proves that the ensemble of excited-state tryptophan residues is homogeneous in all cases. From the time- and spectrally-resolved fluorescence data we extracted the Dynamic Stokes Shift (DSS) and the functional form for the decay of the excited-state population. The DSS is different between E21W and K58W, but for each variant the DSS is identical in both solvents, which suggests that the relaxation dynamics of the protein matrix is unaffected by deuterium substitution. The excited-state population decay for E21W is unaffected upon isotopic substitution, but for K58W the decay is considerably faster in H_2O than in D_2O. Considering that the local concentrations of H^+ and D^+ will be low near R165 (and hence W21) but high near E121 (and hence W58), we suggest that the observed difference in the decay rates for K58W is explained by the fact that H^+ quenches tryptophan fluorescence more efficiently than D^+.

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968-Plat How To Analyse Protein Fluorescence Spectra?

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Fluorescence spectroscopy is a powerful tool for the investigation of protein structure, conformations and dynamics since fluorescence properties of tryptophan residues vary widely depending on the tryptophan environment in a given protein. The major goal in the application of tryptophan fluorescence spectroscopy is to interpret the fluorescence properties in terms of structural parameters and to predict of the structural changes in the protein. We have developed methods for the mathematical analysis of fluorescence spectra of multitryptophan proteins aimed at revealing the spectral compo-

nents of individual tryptophan or clusters of tryptophan residues located close to each other. Also, we have created an algorithm for the structural analysis of the tryptophan environment in 3D atomic structures of proteins from Protein Data Bank (PDB). The successful design of the methods of spectral and structural analysis opened an opportunity for establishing a relationship between the spectral and structural properties of a protein. We have integrated the developed software modules, introduced a new program for the assignment of tryptophan residues to spectral-structural classes, and created a web-based toolkit PFAST: Protein Fluorescence and Structural Toolkit. PFAST contains 3 modules:

1. FCAT is a fluorescence-correlation analysis tool, which decomposes protein fluorescence spectra to reveal the spectral components of individual tryptophan residues or groups of tryptophan residues located close to each other, and assigns spectral components to one of five previously established spectral-structural classes.
2. SCAT is a structural-correlation analysis tool for the calculation of the structural parameters of the environment of tryptophan residues from the atomic structures of the proteins from the PDB, and for the assignment of tryptophan residues to one of five spectral-structural classes.
3. The last module is a PFAST database that contains protein fluorescence and structural data obtained from results of the FCAT and SCAT analyses.

Symposium 11: Collective Motor Dynamics in Cell Division

969-Symp Light microscopy of kinetochore protein architecture and mechanisms achieving spindle bipolarity

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The kinetochore is a protein assembly that links centromeric DNA and the plus ends of spindle microtubules. More than 70 different proteins within 12 or more protein complexes are now known to contribute to the core linkage and many are highly conserved from yeast to man. Their architecture within the kinetochore is poorly understood because the kinetochore core is only about 100 nm thick, about half the Abbe limit of resolution for light microscopy, and because it is has been very difficult to obtain molecular specificity at the higher resolution provided by electron microscopy. We have developed a light microscopy method for obtaining the relative distance between two different color fluorescent probes that label different protein domains at kinetochores. This method called "kinetochore-speckle high resolution co-localization" (K-SHREC) has an accuracy near 10 nm when applied to metaphase cells when sister kinetochores become aligned in opposite directions by pulling forces toward opposite poles. A 3-D Gaussian fitting algoithum is used to find the centroid coordinates of objective point spread function (PSF) images of red and green fluorescently labeled protein domains . The difference, Delta, between centroids is computed after correcting for chromatic aberrations. We are building-up the protein architecture of human and budding yeast kinetochores by K-SHREC measurements relative to the special CENP-A histone within centromeric nucleosomes at the inner kinetochore and the microtubule attachment site at the N-terminus of the Ndc80 complex within the outer kinetochore.

Spindle bipolarity is critical for achieving accurate chromosome segregation. Micromanipulation of meiotic spindles and asters assembled within Xenopus egg extracts has revealed multiple ways that the microtubule motor cytoplasmic dynein functions in achieving spindle bipolarity. Supported by NIH GM 24364.

970-Symp Large-Scale Coordination of Actin Polymerization Forces

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Many complex cellular processes, from mitosis to cell motility, depend on the ability of the cytoskeleton to generate force, either through polymerization and depolymerization of actin or microtubules or through the action of motor proteins. Large-scale cell behavior relies on coordination of cytoskeletal forces with other mechanical elements including cell-substrate adhesions and membrane tension. We have examined the mechanisms of cellular force coordination in the crawling motility of fish epidermal keratocytes. These simple-shaped cells are notoriously well-coordinated, and are able to maintain essentially constant cell shape, speed and directional persistence during rapid motility. Nevertheless, individual keratocytes demonstrate a variety of shapes and speeds. We performed detailed quantitative and correlative analysis of cell morphology and movement on a large number of cells, and used this extensive data set as a basis for the development of a mathematical model that relates the molecular-level dynamics of actin network treadmilling and the forces imposed on this network by the cell membrane to the large-scale geometry of the cell. Our model is able to quantitatively explain the main features of keratocyte shapes, and predict the relationship between cell geometry and speed. Surprisingly, we do not find a significant role for the contractile activity of myosin II in cell body translocation or in trailing edge retraction; instead, the major contribution of myosin II activity in these cells appears to be in assisting actin network disassembly. The dynamic coupling between membrane tension, cytoskeletal assembly/disassembly dynamics, and motor protein activity that we have documented in keratocyte motility is likely to contribute to other large-scale actin-dependent cell shape changes including cytokinesis.

971-Symp Non-equilibrium Dynamics of the Cytoskeleton

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Networks of filamentous proteins play a crucial role in cell mechanics. These *cytoskeletal* networks, incorporating various cross-linking and other associated proteins largely determine the (visco)