1926-Plat
Compensation Of Tissue-induced PSF Aberrations Using Adaptive Phase Modulation
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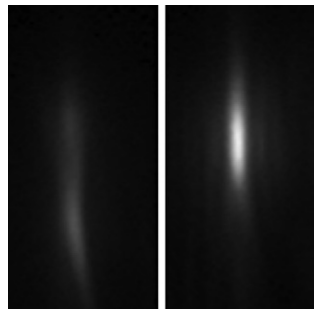

Tissue structures present index mismatches at a variety of spatial scales that can aberrate the focal volume and thus blur cellularly resolved multiphoton images acquired within biological tissues and live animals. We are surveying the types of aberrations that are caused by a variety of tissues to determine the best phase modulation strategies for adaptively correcting the excitation wavefront. A Ti:Sapphire beam is reflected off of a reflective spatial light modulator conjugate to the objective pupil plane. The excitation point-spread-function (PSF) is directly imaged with a separate objective mounted laterally to the optic axis. We find that the fluorescence signal increases with increasing size of the scattering structures. Resolution degradation, however, reaches a maximum with scatterer spatial frequencies at one tenth of the maximal frequency allowed by the focusing objective NA. PSF aberrations can be somewhat compensated by modulating the phase at the back aperture using Zernike polynomials as a basis set for increasing overall image brightness. (See figure for uncorrected vs corrected PSF's through mouse peritoneum.) Initial results show that spherical aberration is a problem, but not the only problem. (Research supported by NIH/NIBIB 41 RR04224 and NIH/NCI R01 CA116583.)

1927-Plat
Orange and Red Fluorescent Protein Optical Highlighters
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Photoconversion of fluorescent proteins (FPs) is finding increased application for routine optical highlighting in live cell imaging and supper-resolution microscopy. Photoconversion involves light-induced shifts in fluorescence properties, which are dependent on the inherent photophysical properties of the FPs. To date, all described photoconvertable FPs have been red-shifting converters. We have recently discovered that photoconversion properties are quite common among well-characterized orange and red FPs (a screen of 12 fluorescent proteins identified 8 variants exhibiting photoconversion behavior), and that several of these fluorescent proteins display a new phenomenon of blue-shifting photoconversion. A major advantage of red-to-green photoswitches is the absence of spectral bleedthrough of the initial fluorescence into the detector channel for the photoconverted species, which permits quantitative imaging of the photoconverted product as well as imaging of the initial fluorescent species using a single excitation wavelength. In addition to the blue-shifted photswitching FPs, another promising discovery from our screen is two orange FP variants that could be efficiently photoconverted to a bright and photostable far-red fluorescent species. We expect these orange optical highlighters to have significant impact for fluorescent probe development, live cell highlighting, as well as super-resolution microscopy. First, the photoconverted red species is the first fluorescent protein found to have an excitation maximum beyond 600nm. Second, photoconversion is induced using blue light, rather than near UV-light, which can greatly enhance live cell applications. Third, the red-shifted spectral properties of both the initial orange and photoconverted red species reduces the potential interference from cellular autofluorescence, and finally, these FPs are well suited for dual-probe optical highlighting applications together with photoactivatable green FPs, like PA-GFP or Dronpa.

1928-Plat
Medical Endoscopes for Multiphoton Microscopy
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Multiphoton laser scanning microscopy (MPM) is a nonlinear optical technique allowing imaging deeper into tissue while avoiding out-of-focus fluorescence and phototoxic stress on living tissues. Near infrared pulsed laser illumination nonlinearly excites the intrinsic tissue fluorescence and second harmonic generation to image the tissues. Medical-Multiphoton Microscopic-Endoscopy (M-MPM-E) can facilitate non-invasive diagnosis of diseased state in situ without resection of tissue, the grand goal of 'optical biopsy'. To enable in vivo medical applications of MPLSM, the development of compact devices is crucial. After much progress in the tabletop MPM over the past two decades, the miniature instrumentation required for endoscopy remains primitive. Here we describe the design of a small lens system suited for the endoscopy of M-MPM-E.
It has been argued previously that a microscope objective lens with high-NA and low magnification should be favorable in deep tissue MPM. We have designed and studied the properties of a reflective objective lens for M-MPM-E. Our optical design includes a modified Schwarzschild objective lens with a raster scanned laser beam, where dichroic thin film coating allows separate propagation of IR excitation and visible light fluorescence collection pathways. By engineering different magnifications for the two different spectra, it is possible to collect the fluorescence efficiently, while maintaining the high-NA for the NIR excitation spectrum. The outer diameter of the lenses of one design are 3.2mm, 0.55-NA, and the field of view is approximately $200\mu m \times 200\mu m$.
Our objective lens demonstrates close to the diffraction-limited performance. Although the central obstruction, a universal undesirable feature of reflective optics, is not avoided in our design, the effect is substantially mitigated in the two-photon point spread function. We are fabricating the device, to be included in the prototype endoscope of 5 mm maximum diameter. Research supported by NIH grant 1-R01-EB006736-02.

Platform AH: Muscle Regulations

1929-Plat
Differences in the Mechanisms of Calcium Regulation of the Acceleration of ADP Dissociation from Myosin-ADP and Myosin-ADP-Pi by Native Cardiac Thin Filaments
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We have used double mixing stopped-flow fluorescence to measure the kinetics of the dissociation of the hydrolysis products deoxymantADP (mdADP) from cardiac myosin-mdADP and cardiac myosin-mdADP-Pi by native cardiac thin filaments. Increasing the calcium concentration increases the rate of dissociation of mdADP from cardiac myosin-S1-ADP-Pi ~100 fold from 0.5 s^{-1} at pCa $>$ 7 to 50 s^{-1} at pCa $<$ 4. Increasing the calcium concentration increases the rate of dissociation of mdADP from cardiac myosin-S1-ADP-Pi only 10 fold from 15 s^{-1} at pCa $>$ 7 to 150 s^{-1} at pCa $<$ 4. These results indicate that slow dissociation of phosphate limits the rate of ADP dissociation from acto(thinfilaments)myosin-ADP-Pi and that there are different mechanisms for the calcium regulation of dissociation of the two products of myosin ATP hydrolysis, ADP and phosphate. These results support a mechanism in which the step of the hydrolysis cycle that is principally regulated by calcium is phosphate dissociation from actomyosin-ADP-Pi and do not support a mechanism such as the three state mechanism in which the regulation is a result different distributions of thin filament states in presence and absence of bound calcium that occur prior to myosin binding. This work is supported by a NIH HL84604.

1930-Plat
Effects of Elevated Solvent Viscosity on Calcium Dependence of Cardiac Myofilament Contractility
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We have previously shown with skinned skeletal muscle fibers at maximum Ca^{2+} activation and unregulated *in vitro* motility assays that solvent viscosity modulates actomyosin function in a manner consistent with diffusional limitation of a kinetic process. To determine whether viscosity influences thin filament regulatory protein dynamics, we performed experiments in cardiac muscle preparations with varying [Ca^{2+}]. First, *in vitro* motility assays were conducted using thin filaments reconstituted with recombinant human cardiac troponin and tropomyosin, and rabbit skeletal HMM and actin; solution viscosity was varied by addition of sucrose. At maximum [Ca^{2+}], we observed that thin filament sliding speed was inversely proportional to the solution viscosity. In addition, Ca^{2+}-sensitivity (pCa_{50}) of thin filament sliding speed decreased significantly with elevated viscosity ($\eta/\eta_0 \geq \sim 1.6$). For comparison with results from unloaded motility assays, single skinned porcine cardiomyocytes were used to measure steady-state isometric force and the kinetics of isometric tension redevelopment (k_{TR}) when viscosity within the myofilament lattice was elevated. Maximum Ca^{2+} activated force changed very little for sucrose $\leq$ 0.3 M ($\eta/\eta_0 \sim 1.4$) or glucose $\leq$ 0.875 M ($\eta/\eta_0 \sim 1.66$), but decreased at higher concentrations. Maximum k_{TR} decreased steeply and monotonically with increased sucrose or glucose. Ca^{2+}-sensitivity of isometric force also decreased in accord with the *in vitro* motility assay results. While either 0.3 M sucrose or 0.875 M glucose lowered k_{TR} at high [Ca^{2+}], there was little or no effect at low [Ca^{2+}]. Taken together, these results suggest that cross-bridge cycling is more affected by elevated viscosity than thin filament dynamics in cardiac muscle; changes in

Ca^{2+}-sensitivity are therefore consistent with the hypothesis that cross-bridges play a key role in cardiac thin filament activation. Supported by: NIH HL63974, GM07592, AHA 0615164B.

1931-Plat

Dynamics of Bi-Functional Labeled Tropomyosin in Muscle Ghost Fiber Monitored by Saturation Transfer EPR

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Tropomyosin (Tm), an alpha-helical coiled-coil protein, is a key regulatory protein in muscle contraction. To date, little is known about the extent of Tm flexibility and the role of Tm dynamics in muscle regulation. In this work, the flexibility of two different regions of Tm was assessed using Saturation Transfer Electron Paramagnetic Resonance (ST EPR). In order to fully immobilize the spin probe on the surface of Tm we used a bi-functional spin label attached to i, i+4 positions of the coiled-coil obtained by cysteine mutagenesis. We have used conventional EPR and ST-EPR to detect wide range of dynamics from the very slow (millisecond) motions to fast sub-nanosecond modes. The labeled Tm mutants were reconstituted into ''ghost muscle fibers'' from which the myosin filaments and intrinsic regulatory proteins (tropomyosin, troponin) were removed.

ST-EPR of the two mid-region mutants Tm H153C/D157C and Tm G188C/E192C as well as the C-terminus mutant Tm A268C/E272 gave a correlation time of 10.5 us $\pm$ 4.5 us, 42.5 us $\pm$ 27.5 us, and 42.5 us $\pm$ 27.5 us respectively (using H''/H and L''/L ratios of V'_2 spectra). The difference in correlation time between the different di-mutants is an indication of the differential flexibility of the Tm protein. The study of the N-terminus (L13C/N17C) di-mutant will give us an additional understanding of Tm flexibility. Finally the introduction of Troponin complex (Tn) as well as S1 head of myosin under high and low calcium concentrations will give a complete picture of the dynamics of Tm in muscle regulation.

1932-Plat

C-terminal Region Of Troponin I Interacts Near Residue 146 Of Tropomyosin In A Ca^{2+} Dependent Manner

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Force generation in striated muscle is initiated by Ca^{2+} binding to troponin C in the actin-tropomyosin-troponin (actinTmTn) thin filament. Potter & Gergely, (*Biochemistry* 1974); have suggested that the inhibitory subunit of Tn, troponin I (TnI) interacts with Tm as well as actin to inhibit contraction in the absence of Ca^{2+}. Zhou et. al., and Geeves et. al., (*Biochemistry* 2000) proposed that this interaction involves a specific site on Tm. Last year (Mudalige, Tao and Lehrer, 52nd annual meeting of Biophysical Society 2008) we reported the formation of a Ca^{2+}-dependent cross-link between a benzophenone-maleimide label at Tm residue 146 and TnI (Tm*146-TnI).

To determine the cross-linking site of TnI with Tm*146, we purified the photochemically cross-linked complex, Tm*146-TnI from uncross-linked proteins using HPLC, and SDS gels and subjected the selected Tm*146-TnI band to in-gel tryptic digestion .

From the comparison of MALDI-TOF spectra of tryptic peptides of in-gel digested Tm*146, TnI and Tm*146-TnI, a new peptide of MW 2601.2 Da was identified. Two possible TnI tryptic peptides which contains the Tm 143-154 tryptic peptide and probe with similar MW were identified: 1) peptide 157-163 (MW 2602.4 Da); 2) peptide 176-182 Met oxidized, (MW 2600.3 Da). A cross-link in either of thee peptides supports the recently published image reconstructions which show the C-terminal domain of TnI interacting with both actin and Tm across the actin filament away from the bulk of the Tn complex (Galinska_Pakoczy et al, JMB, 2008). Our identification of the cross-linked residue on TnI (in progress), will further localize Tn on the actinTm muscle thin filament in the absence of Ca^{2+} (Supported by NIH HL 22461).

1933-Plat

Impaired Myofilament Contractility in Post-infarct Remodeled Myocardium is Restored upon β-Adrenergic Stimulation

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Previously we have shown that *in vivo* cardiac responsiveness to exercise-induced increases in noradrenaline was blunted in pigs with a myocardial infarction (MI), consistent with defects in β-adrenergic signaling. Here we tested the hypothesis that the blunted increase in pump function with exercise after MI is due to reduced myofilament responsiveness, and is prevented by β-blocker therapy. In pigs with a MI induced by ligation of the left circumflex coronary artery, β-blocker therapy (bisoprolol, MI+β) was initiated on the first day after MI. Myofilament force measurements and protein analysis were performed in left ventricular subendocardial biopsies taken at baseline, and upon dobutamine stimulation 3 weeks after MI or sham (n=6). Isometric force was measured in single permeabilized cardiomyocytes. At baseline, maximal force (Fmax) was lower in MI compared to sham, while Ca^{2+}-sensitivity (pCa_{50}) was higher (both $P<0.05$). Passive force (Fpas) did not differ. Fmax did not change upon dobutamine in sham, while it markedly increased in MI. Moreover, the dobutamine-induced decrease in pCa_{50} was larger in MI than in sham. Beta-blockers prevented baseline myofilament dysfunction, reduced Fpas and enhanced the responsiveness to β-AR stimulation illustrated by a large change in pCa_{50} upon dobutamine. Baseline phosphorylation of β-adrenergic target proteins (myosin binding protein C and troponin I) was not altered in MI, while the dobutamine-induced increase in troponin I phosphorylation was less in MI compared to sham and MI+β. Dobutamine enhanced myosin light chain 2 phosphorylation solely in sham. In conclusion, acute β-adrenoceptor stimulation largely restores baseline myofilament dysfunction despite attenuation of β-adrenergic-mediated troponin I phosphorylation. Myofilament dysfunction in remodelled myocardium and its reversal by β-blockers is not a direct consequence of reduced PKA-mediated phosphorylation, and does not contribute to the blunted in vivo response to β-adrenoceptor stimulation.

1934-Plat

Reference Free Single Particle Analysis Of Reconstituted Thin Filaments

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A detailed three-dimensional structure of the muscle thin filament is required in order to understand its regulation. To this end we have applied a reference free single particle analysis approach to electron microscope images of negatively stained reconstituted thin filaments from skeletal actin and cardiac tropomyosin and troponin. The filaments were prepared in a low Ca^{2+} buffer. For image analysis the filaments were segmented into ~800Å long particles centred on the troponin complex. Density attributable to troponin and tropomyosin is readily identifiable in the two-dimensional class averages and the three-dimensional reconstruction. The data have previously been analysed using a model-based single particle method (Pirani *et al.*, 2005, 2006). Our non-model based approach and novel strand averaging procedure has enabled us to quantify directly the stagger or axial rise between adjacent troponin complexes (~27.7Å). Comparison with our previous analysis of native thin filaments indicates that reconstituted filaments assemble with the same arrangement of troponin as in vivo, *viz* .in register on both helical strands with a ~40 nm repeat. This indicates that troponin and tropomyosin can organise themselves on actin filaments without requiring any other sarcomeric proteins.

Pirani A., Vinogradova M.V., Curmi P.M., King W.A., Fletterick R.J., Craig R., Tobacman L.S., Xu C., Hatch V., Lehman W. 2006. An atomic model of the thin filament in the relaxed and Ca^{2+}-activated States. J Mol Biol 357(3):707-17.

Pirani A., Xu C., Hatch V., Craig R., Tobacman L.S., Lehman W. 2005. Single particle analysis of relaxed and activated muscle thin filaments. J Mol Biol 346(3):761-72.

1935-Plat

Calcium-Regulated Conformational Changes in the COOH-terminus of Troponin I

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The troponin complex plays an essential role in the calcium regulation of skeletal and cardiac muscle contractions. Of the three subunits of troponin (TnC, TnI and TnT), TnI is the inhibitory subunit that responds to the binding of Ca^{2+} to TnC during the activation of contraction. The COOH-terminal region of TnI is a highly conserved structure implying a fundamental function. Previous studies using reconstituted troponin or myofilaments suggested that the COOH-terminal domain of TnI undergoes epitopic and positional changes in the presence or absence of calcium. Here we tested the calcium-induced conformational changes in the COOH-terminal region of TnI by engineering a unique Cys at the COOH terminus of TnI for the addition of a reporting label. Monoclonal antibody epitope analysis and protein binding assays indicated that this modification and the replacement of two internal Cys residues (C81I and