Sarah Rice, Northwestern University

Moving Towards "Systems Biophysics" with Molecular Motors

The abstracts presented on motor mechanism and force generation at this Biophysical Discussions demonstrate that molecular motors are a particularly exciting area of study in this era of systems biology. As cell biologists are combining genomic analysis, RNA interference, biochemistry, and new imaging techniques on molecular motor-driven systems, biophysicists are quickly obtaining mechanistic information about the molecular motor components of those systems with unprecedented accuracy. This dual approach, highlighted by the research presented in this session, is rapidly expanding our understanding of the molecular motor systems that drive active movement in living organisms. The studies presented here describe the "original" motor-driven system, muscle, as well as a variety of others such as the machinery of cell division, the bacterial flagellar motor (**Xing, #75**), the chloroplast (**Varco-Merth, #102**) and even the extracellular matrix **(Goldberg, #86)** while striving for the detailed understanding of these systems and their components that molecular motor biophysicists have always expected of themselves.

This year, the cell division apparatus provides a particularly good example of a system whose intricacies are being elucidated by both *in vivo* studies of the mitotic apparatus and by theoretical and biophysical studies of its components. **Grischuk and colleagues (#111)** present a broad set of experiments aimed at dissecting the mechanism of force generation in the mitotic spindle. These researchers began by asking how much force can be generated by microtubule depolymerization. They approached this question using laser trap experiments (Grishchuk et al., 2005) and by theoretical models of microtubule properties (Molodtsov et al., 2005). Their finding that the depolymerization of a single microtubule generates roughly 10 times the force of a cytoskeletal motor indicates that microtubule depolymerization may provide most of the force for chromosome movement during mitosis. These researchers have now followed these studies up with experiments examining poleward chromosome motion in yeast. They found that deletion of any one of the minus-end directed molecular motors involved in poleward movement of chromosomes does not abrogate that movement, though these motors do help to orient chromosomes and control the length of kinetochore fibers. These studies indicate that the molecular motors orient chromosomes and assist in the organization and structure of the spindle, while the actual force that moves chromosomes poleward is exerted by the microtubule depolymerization "motor". Studies on several other critical motor components of the mitotic apparatus are being presented in posters here as well, including CENP-E (**van Duffelen #149**), the Dam1 kinetochore ring complex (**Asbury #47**), Klp10A (**Sharp #172**), Eg5, and dynein.

The 6 posters on Eg5 mechanism and 9 dynein posters presented this year are of outstanding interest, and by their numbers they reflect our rapidly increasing understanding of these two important proteins. Eg5 is the target of Monastrol and similar inhibitors, which are the first promising antimitotic drugs that do not target microtubules. Here **Krzysiak** (**#58 and #68**) and **Valentine #91,** along with their colleagues, present biochemical, cryo-EM, and force clamping data on a stable dimeric form of Eg5. These

studies have allowed for direct comparison of Eg5's properties with those of conventional kinesin. Like conventional kinesin, an Eg5 dimer exhibits alternating site catalysis, binds to two adjacent tubulin heterodimers on the same microtubule protofilament, and takes 8-nm steps. However, Eg5 is a slower motor with higher affinity for ATP, and its movement is less sensitive to applied load than that of conventional kinesin. It remains to be determined whether this is a specific adaptation to the task of spindle assembly and maintenance, but the idea seems very plausible. These researchers also have cryo-EM structural data indicating that the Eg5 inhibitor Monastrol weakens the microtubule interaction of the stable Eg5 dimer. Importantly, this data is a first structural glimpse of a small molecule allosteric effector of the interaction of a cytoskeletal motor and its partner filament.

Despite its great importance in biological motility, dynein has been relatively intractable for biophysical and mechanistic studies until a recent, landmark paper by Reck-Peterson and colleagues (Reck-Peterson et al., 2006). In a tour-de-force, these researchers purified a minimal processive dynein motor from yeast that has enabled detailed force and velocity measurements (presented by **Reck-Peterson #114** and **Gennerich #115)** as well as mutagenic analysis of dynein's components (presented by **Carter #150).** Armed with these basic tools, these researchers have found that yeast cytoplasmic dynein is a processive dimer and does not require other dynein-associated proteins for its processivity. Further dissecting dynein's mechanism, they found that a 26 amino acid section of the linker region connecting the AAA+ core of the motor to the microtubule-binding coiled-coil stalk is critical for both motility and ATPase activity. This is consistent with previous electron microscopy and single particle reconstruction data indicating that this linker may be dynein's lever arm (Burgess et al., 2003). However, dynein is starkly different from kinesin and myosin, and predictably, its motile mechanism appears to be complex. Constructs with truncated linker regions exhibit limited motility, indicating that regions of dynein other than the linker contribute to its movement on microtubules. Furthermore, dynein has a variable, load-dependent stepping pattern and step size distribution, unlike other known processive kinesins and myosins. The length of the linkage between the two heads of a dynein dimer appears to be a critical determinant of this stepping behavior. These studies are complemented by crosslinking data (presented by **Kon #141)**, showing that crosslinking the coiled-coil region between the AAA+ and microtuble-binding regions can trap dynein either in a state with high, "activated" or low, "basal" ATPase activity. This is an exciting time for dynein. The basic mechanisms of the other two linear molecular motor superfamilies (kinesins and myosins) have been modeled and drawn in great detail (see, for example, the movies at http://valelab.ucsf.edu/movies/moviesovr.html). These researchers at last have the tools to generate a similar picture of dynein's unique molecular mechanism. This will be critical to our understanding of molecular motor-driven systems, where dynein provides the vast majority of movement to the minus ends of microtubules.

Together, dynein and myosin V have been a focus of recent work on cargo binding and motor coordination. Not presenting a poster at this meeting are Pashkova and colleagues, who solved the X-ray crystal structure of the myosin V tail (Pashkova et al., 2006). This structure revealed different stretches of residues in the tail that are

required for secretory and vacuolar movement, respectively, in yeast. Following this study, structures were solved of myosin V in a putative regulated conformation by two other groups, Liu and colleagues and Thirumurugan and colleagues (Liu et al., 2006; Thirumurugan et al., 2006). Presented here are other potential control mechanisms of both myosin V and myosin VI, in particular the response of the ATPase activity of these myosins to external loads (**Oguchi #146** and **Mikhailanko #78**). Both of these motors are distinct from myosin II in that ADP release is rate-limiting, and it is this property that enables these two myosins to be processive. The affinity of both motors for ADP and hence their processive movement is load-dependent. The biological significance of these load-dependent control mechanisms remains for further study but is potentially high. Myosin VIB in particular functions to anchor the apical hair cell membrane to actin filaments in the cuticular plate, and therefore may exist primarily in a rigor state *in vivo*.

As always, the Biophysical Discussions feature a number of newly developed and greatly improved techniques for studying molecular motors. This year, spectroscopic and electron microscopic studies are increasing both the breadth and resolution of data available on molecular motor systems. This has led to some surprising new findings on an old system, namely, muscle. **Berger and colleagues (#116)** used two different tryptophan mutants in smooth muscle myosin II to correlate nucleotide-dependent changes in the active site with the opening and closing of the actin-binding cleft. Contrary to what is generally believed but consistent with recent results (Robertson et al., 2005; Yengo et al., 2002), nucleotide-dependent changes in the active site do not appear to be directly coupled to changes in the actin cleft. Another poster presented here shows that the conformation of the actin cleft and ADP affinity of fast skeletal myosin depends on light chain phosphorylation **(Cooke, #132)**. Higher resolution electron microscopy data is also presented on both the actomyosin (**Schroeder, #126**) and kinesin-microtubule complexes (**Sindelar #40 and Hirose #134**). In the case of kinesin, these studies have revealed previously undescribed nucleotide-dependent structural changes that may help to explain kinesin's microtubule affinity in different nucleotide states.

The last paper to be highlighted by this summary represents a worthy goal of this research. **Hartman #129** presents data on the mechanism of a small molecule activator of cardiac myosin, CK-1213296. This drug appears to increase cardiac function and force production in dog models and is a potentially useful intervention for patients experiencing cardiac infarct. In the process of learning the intricate details of molecular motor systems, useful drug compounds are being developed for medical intervention. The compounds themselves will drive more breakthroughs in research, and so on. Molecular motor "systems biophysics" is coming to fruition.

Published References

Burgess, S.A., M.L. Walker, H. Sakakibara, P.J. Knight, andK. Oiwa. 2003. Dynein structure and power stroke. *Nature* 421(6924):715-718.

Grishchuk, E.L., M.I. Molodtsov, F.I. Ataullakhanov, andJ.R. McIntosh. 2005. Force production by disassembling microtubules. *Nature* 438(7066):384-388.

Liu, J., D.W. Taylor, E.B. Krementsova, K.M. Trybus, andK.A. Taylor. 2006. Three-dimensional structure of the myosin V inhibited state by cryoelectron tomography. *Nature* 442(7099):208-211.

Molodtsov, M.I., E.L. Grishchuk, A.K. Efremov, J.R. McIntosh, andF.I. Ataullakhanov. 2005. Force production by depolymerizing microtubules: a theoretical study. *Proc Natl Acad Sci U S A* 102(12):4353-4358.

Pashkova, N., Y. Jin, S. Ramaswamy, andL.S. Weisman. 2006. Structural basis for myosin V discrimination between distinct cargoes. *Embo J* 25(4):693-700.

Reck-Peterson, S.L., A. Yildiz, A.P. Carter, A. Gennerich, N. Zhang, andR.D. Vale. 2006. Single-molecule analysis of dynein processivity and stepping behavior. *Cell* 126(2):335-348.

Robertson, C.I., D.P. Gaffney, 2nd, L.R. Chrin, andC.L. Berger. 2005. Structural rearrangements in the active site of smooth-muscle myosin. *Biophys J* 89(3):1882-1892.

Thirumurugan, K., T. Sakamoto, J.A. Hammer, 3rd, J.R. Sellers, andP.J. Knight. 2006. The cargo-binding domain regulates structure and activity of myosin 5. *Nature* 442(7099):212-215.

Yengo, C.M., E.M. De La Cruz, L.R. Chrin, D.P. Gaffney, 2nd, andC.L. Berger. 2002. Actin-induced closure of the actin-binding cleft of smooth muscle myosin. *J Biol Chem* 277(27):24114-24119.

Session: Force-generating mechanisms

The beginning of kinesin's force-generating cycle visualized at 9A resolution

Charles V. Sindelar, **Ph.D.**, Kenneth Downing, Ph.D..
Life Sciences Division, Lawrence Berkeley National Labs, Berkeley, CA, USA.

After encountering the microtubule, kinesin releases a substrate ADP and subsequently strengthens its attachment to the microtubule 100-fold. However, no relevant change has yet been found in kinesin's microtubule-binding interface before and after this ADP release. To investigate the affinity enhancement, we have solved the 9-Angstrom cryo-EM structure of nucleotide-free kinesin complexed with the microtubule. The high resolution of our structure, which was achieved by single particle methods using native, asymmetric 13-protofilament microtubules, enabled us to visualize individual alpha helices and the large central beta sheet of kinesin as well as tubulin's GTP and taxol ligands. Both of kinesin's switch domains ("switch I" and "switch II"), which are thought to coordinate its ATP-sensing and microtubule-binding functions, are in new conformations compared to crystal structures of our construct. These conformational changes allow the nucleotide-free kinesin monomer to reorient on the microtubule relative to previously reported ATP- and ADP-bound microtubule complexes of monomeric kinesin.

The Dam1 kinetochore ring complex harnesses microtubule dynamics to produce force and movement

Charles L. Asbury[1], Trisha N. Davis[2], Daniel R. Gestaut[2], Andrew F. Powers[1], Andrew D. Franck[1].
[1]Physiology and Biophysics, University of Washington, Seattle, WA, USA, [2]Biochemistry, University of Washington, Seattle, WA, USA.

Kinetochores remain attached to microtubule tips during mitosis even as the tips assemble and disassemble under their grip, allowing filament dynamics to produce force and move chromosomes. The specific proteins mediating tip attachment are uncertain, and the mechanism of microtubule-dependent force production is unknown. Recent work suggests the Dam1 complex, an essential component of kinetochores in yeast, may contribute directly to kinetochore-microtubule attachment and force production, perhaps by forming a sliding ring that encircles the microtubule. To test these hypotheses, we developed an *in vitro* motility assay where beads coated with pure recombinant Dam1 complex were bound to the tips of individual dynamic microtubules. The Dam1-coated beads remained tip-bound and underwent assembly- and disassembly-driven movement over ~3 μm, comparable to chromosome displacements *in vivo*. Dam1-based attachments to assembling tips were robust, supporting 0.5 - 3 pN of tension applied with a feedback-controlled optical trap as the microtubules lengthened ~1 μm. The attachments also harnessed energy from microtubule disassembly to generate movement against applied tension. Reversing the direction of force (i.e., switching to compressive force) caused the attachments to disengage the tip and slide over the filament, but sliding was blocked by areas where the MT was anchored to a coverslip. These results provide direct evidence for a coupling structure encircling the filament and demonstrate how the Dam1 complex can contribute to microtubule-driven chromosome movement.

Poster 3

A structural model for monastrol inhibition of dimeric kinesin Eg5

Troy C. Krzysiak[1], Thomas Wendt[2], Lisa R. Sproul[1], Heinz Gross[3], **Susan P. Gilbert**[1], Andreas Hoenger[4].
[1]Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA, [2]European Molecular Biology Laboratory, Heidelberg, Germany, [3]Electron Microscopy ETH Zurich (EMEZ) c/o Institution for Applied Physics, Zuerich-Hoenggerberg, Switzerland, [4]University of Colorado at Boulder, Boulder, CO, USA.

Eg5 or KSP is a homotetrameric Kinesin-5 involved in centrosome separation and assembly of the bipolar mitotic spindle. Analytical gel filtration of purified protein and cryo-electron microscopy (cryo-EM) of unidirectional shadowed microtubule-Eg5 complexes have been used to identify the stable dimer Eg5-513. The motility assays show that Eg5-513 promotes robust plus-end-directed microtubule gliding at a rate similar to that of homotetrameric Eg5 *in vitro*. Eg5-513 exhibits slow ATP turnover, high affinity for ATP, and a weakened affinity for microtubules when compared to monomeric Eg5. We show here that the Eg5-513 dimer binds microtubules with both heads to two adjacent tubulin heterodimers along the same microtubule protofilament. Under all nucleotide conditions tested, there were no visible structural changes in the monomeric Eg5-microtubule complexes with monastrol treatment. In contrast, there was a substantial monastrol effect on dimeric Eg5-513, which reduced microtubule lattice decoration. Comparisons between the X-ray structures of Eg5-ADP and Eg5-ADP-monastrol with rat kinesin-ADP after docking them into cryo-EM 3-D scaffolds revealed structural evidence for the weaker MT-Eg5 interaction in the presence of monastrol.

Dimeric Eg5/KSP Exhibits Alternating Site ATP Catalysis

Troy C. Krzysiak, B.S, Susan P. Gilbert, Ph.D..
Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA.

Eg5/KSP is a member of the Kinesin-5 (BimC) family of homotetrameric motors. These N-terminal kinesins provide a plus-end-directed force associated with sliding microtubules during centrosome separation to assemble the bipolar spindle. To define the cooperative interactions between the motor heads and the microtubule, a dimeric motor was engineered. This Eg5 dimer is processive with its motor heads binding along a single protofilament (Valentine et al. 2006. *Nature Cell* Apr 2 ePub ahead of print; Krzysiak et al. 2006. *EMBO J*. In press). The steady-state and presteady-state kinetics show that ATP binding occurs as a two-step process with an ATP-dependent isomerization at 50 s^{-1} which forms the ATP-hydrolysis competent intermediate. Single-turnover experiments were required to detect a presteady-state burst of ATP hydrolysis. At ATP concentrations in which only one head participates, the rate was ~5 s^{-1}; however, at ATP concentrations where both heads of the dimer participate, the exponential rate is observed at approximately half. In addition, the kinetics of ATP-promoted microtubule dissociation and mantADP release show an initial fast phase followed by a second slow phase with observed rates of the second phase more similar to steady-state turnover. These kinetics are consistent with an alternating cycle of ATP turnover in which the active sites are coordinated to establish processive stepping along the microtubule.
Supported by NIH GM54141 & K02-AR47841.

The torque-speed relationship of the bacterial flagellar motor

Jianhua Xing, **Ph. D.**[1], Fan Bai[2], Richard Berry[2], George Oster[3].
[1]Physical Biosciences Institute, Lawrence Livermore National Laboratory, Livermore, CA, USA, [2]Physics, University of Oxford, Oxford, United Kingdom, [3]University of California, Berkeley, CA, USA.

Many swimming bacteria are propelled by flagellar filaments driven by a rotary motor. Each of these tiny motors can generate an impressive torque. The motor torque vs. speed relationship is considered one of the most important measurable characteristics of the motor and so is a major criterion for judging models proposed for the working mechanism. Here we give an explicit explanation for this torque-speed curve. The same physics can also explain certain puzzling properties of other motors. We also present recent studies on steps of the flagellar motor.

Regulation of Myosin VI's ADP Affinity by Load

Sergey Mikhailenko[1], Yusuke Oguchi[1], Adrian O. Olivares[2], Enrique M. De La Cruz[2], Shin'ichi Ishiwata[1].
[1]Department of Physics, School of Science and Engineering, Waseda University, Tokyo, Japan, [2]Molecular Biophysics & Biochemistry Department, Yale University, New Haven, CT, USA.

Myosin VI "walks" processively along an actin filament toward its pointed end with long (~36 nm) steps. Processive movement of the dimeric myosins has been shown to proceed via the "hand-over-hand" mechanism, which postulates that from the state of a double-headed binding to actin, the trailing head dissociates and swings forward to take the leading position, where it binds to actin before the other head detaches. Therefore, for the efficient processive movement there should exist an asymmetry between two identical heads, each time forcing the trailing head, rather than the leading head, to dissociate from actin more readily than the leading head. We reported at this year's Annual Meeting of the Biophysical Society that the external load applied to the actin-myosin VI bond affects the motor's affinity for ADP, and this effect depends on loading direction. This finding supports a model, in which the internal strain arising from the double-headed binding to actin regulates the biochemical kinetics in the two heads, inducing an asymmetry between them and ensuring a highly effective processive stepping. Here we studied the regulation of myosin VI's affinity for ADP in more detail, revealing the contribution of the on-axis and off-axis strain, by studying the mechanical properties of a single myosin VI-actin complex. A plastic bead with a single attached myosin VI molecule was trapped by optical tweezers, brought in contact with an actin filament, which was immobilized on the glass surface via the biotin-streptavidin interaction, and the stage was then slowly moved with a constant speed, exerting an external load on the myosin-actin bond, towards either pointed or barbed end of the actin filament. The implications of the obtained data for the model of myosin's processivity will be discussed.

Diffusion of Matrix Metalloproteases on the Surface of Collagen Fibrils: The Mobile Cell Surface - Collagen Substratum Interface Hypothesis.

Gregory I. Goldberg, PhD[1], Ivan E. Collier, PhD[2], Saveez Saffarian, PhD[3], Elliot Elson, PhD[4].
[1]Medicine, Biochemistry and Molecular Biophysics, Washington University School of Medicine, Saint Louis, MO, USA, [2]Medicine, Washington University School of Medicine, Saint Louis, MO, USA, [3]Cell Biology, Harvard Medical School, Boston, Ma, MA, USA, [4]Biochemistry and Molecular Biophysics, Washington University School of Medicine, Saint Louis, MO, USA.

We have shown that activated Collagenase (MMP-1) moves processively on collagen fibrils as a “burnt bridges” Brownian Ratchet where the biased component of the motion depends on the proteolysis of collagen but not ATP hydrolysis. A model for a propulsion mechanism coupling the free energy from collagen proteolysis and fibril unfolding to the enzyme motion predicts that a motor consisting of an enzyme dimer could have a 5pN stall force, sufficient for it to be biologically significant. Now we demonstrate that two closely related MMPs, -2 and -9, can diffuse on collagen fibrils and that dimerization of MMP-9 renders it immobile. Most interestingly, diffusion of these enzymes is not blocked by complex formation with TIMP inhibitors despite the fact that the inhibitors occupy a large portion of the solvent exposed surface of the C-terminal domain that is necessary for diffusion. These findings imply that the cell surface tri-molecular activation complex of MT1-MMP/TIMP-2/MMP-2 that we described earlier is mobile relative to the underlying collagen substratum and comprises an essential part of a mobile cell surface - ECM interface that we now propose.

Individual dimers of the mitotic kinesin motor Eg5 step processively and support substantial loads *in vitro*

Megan T. Valentine, Ph.D.[1], Polly M. Fordyce[2], Troy C. Krzysiak[3], Susan P. Gilbert[3], Steven M. Block[4].
[1]Biological Sciences, Stanford University, Stanford, CA, USA, [2]Physics, Stanford University, Stanford, CA, USA, [3]Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA, [4]Biological Sciences & Applied Physics, Stanford University, Stanford, CA, USA.

We probed the mechanochemistry of Eg5, a kinesin-related motor essential for bipolar spindle assembly and maintenance during mitosis, using an optical trapping apparatus and novel motility assay. Individual dimers formed by a recombinant human construct, Eg5-513-5His, stepped processively along microtubules in 8-nm increments against loads as high as 7 pN. Run lengths were short, averaging about eight steps. By varying the applied load and ATP concentration, we determined that the velocity of Eg5 was slower and less sensitive to force than that of conventional kinesin. These differences may arise from distinct mechanical optimizations for motors that work in ensembles during spindle assembly as compared to those that work in isolation during vesicle transport.

The Rotor Ring of Chloroplast ATP Synthase

Ben Varco-Merth, **PhD Candidate**.
Chemistry and Biochemistry, Arizona State University, Tempe, AZ, USA.

ATP synthase couples the transmembrane proton potential produced by mitochondria, chloroplasts and bacteria to the synthesis of ATP from ADP and inorganic phosphate. It is composed of two molecular rotary engines_F_0 (ab_2c_{10-15}), which is fueled electrically by proton flux, and F_1 ($\alpha_3\beta_3\gamma\delta\varepsilon$), which is fueled chemically by ATP hydrolysis. During ATP synthesis, stepwise protonation of a conserved carboxylate on each member of an oligomeric ring of 10-15 c-subunits drives rotation of the rotor moiety ($c_{10-15}ge$) relative to the stator moiety ($a_3b_3dab_2$), driving ATP synthesis.
Here, the crystallization of an oligomeric form of subunit c, the "rotor ring" of F_0, from the spinach chloroplast enzyme is described. Though experimental phases have not yet been determined, analysis of the native diffraction data clearly shows the presence of 14 subunits, in agreement with AFM results. This reveals an apparent symmetry mismatch between the F_1 and F_0 motors and a non-integer proton-to-ATP ratio. This work will examine possible explanations for the symmetry mismatch in this essential biological machine.

Mechanisms of poleward chromosome movement

Ekaterina L. Grishchuk[1], Maxim I. Molodtsov[1,2], Artem K. Yefremov[1,2], Ilia S. Spiridonov[1,2], Fazly I. Ataullakhanov[2,3], J. Richard McIntosh[1].
[1]MCD Biology, University of CO, Boulder, CO, USA, [2]Natl. Res. Cntr. Hematology, Moscow, Russian Federation, [3]Physics Dept., Moscow State Univ., Moscow, Russian Federation.

Poleward chromosome movement during mitosis is dependent upon the activities of minus-end directed, microtubule-dependent motors, and it requires the depolymerization of microtubules. To learn the respective roles of these factors we have taken two approaches. Microtubule depolymerization is studied in a novel experimental system, which allows the direct measurement of the depolymerization force. By conjugating glass microbeads to microtubules through strong inert linkages, and by using laser tweezers we have shown that depolymerizing microtubules can exert a brief tug on an attached bead (Grishchuk et al, *Nature 2005*). Analysis of these interactions with a molecular-mechanical model of microtubule (Molodtsov et al, *PNAS 2005*) suggests that a single disassembling microtubule can generate about ten times the force that is developed by a motor enzyme. Thus, this mechanism might be the primary force for chromosome motion. This experimental system is being applied to analyze force production by depolymerizing microtubules that are linked to cargo via processive couplers.
We are also studying the roles of minus-end directed motors in poleward chromosome motions in vivo using fission yeast (Grishchuk and McIntosh, *submitted*). This organism has three such motors: dynein and two kinesin-14s, Pkl1p and Klp2p. The maximum rate of poleward kinetochore movement was unaffected by the deletion of any of these motors, suggesting that microtubule depolymerization can cause such movements *in vivo*. However, Klp2p, which localizes to kinetochores, contributed to the effectiveness of poleward movement by promoting the shortening of kinetochore fibers. Pklp1 and dynein were required for efficient chromosome bi-orientation. In *pkl1Δ*, whose product normally localizes to the spindle and poles, the checkpoint that monitors chromosome bi-orientation was defective, leading to premature anaphase. Electron microscopy suggests that Pkl1p contributes to error-free bi-orientation by promoting the normal organization of spindle poles, while dynein helps to anchor microtubule minus ends.

Requirements for Yeast Cytoplasmic Dynein Processivity

Samara L. Reck-Peterson, **PhD**, Andrew Carter, PhD, Arne Gennerich, PhD, Ronald Vale, PhD.
Cellular and Molecular Pharmacology, UCSF, San Francisco, CA, USA.

Cytoplasmic dynein is responsible for nearly all minus-end directed microtubule based traffic in most eukaryotic cells and has additional roles in mitosis; however, its mechanism of movement is poorly understood compared to kinesin and myosin. We have developed a method to purify native cytoplasmic dynein and a minimal functional motor domain from S. cerevisiae. Using total internal reflection microscopy, we find that yeast cytoplasmic dynein is a highly processive motor with a run length of ~2 um. Cytoplasmic dynein processivity requires a dimer of two motor domains. As evidence, we find that monomeric dynein is not processive, but processivity can be restored by constitutive (GST) or chemically-controlled (FRB-rapamycin-FKBP12) dimerization. To further examine the minimal requirements for processivity we have made truncations in the dynein tail domain and find that 1/3rd of the dynein tail domain is not required for processive movement. We have also examined the role of the dynein associated proteins, including the dynein light intermediate and intermediate chains and the lissencephaly 1 protein, and find that none of the dynein associated subunits are required for processivity.

Force-production and stepping-mechanism of cytoplasmic dynein

Arne Gennerich, **Ph.D.**, Andrew Carter, Ph.D., Sam Reck-Peterson, Ph.D., Ahmet Yildiz, Ph.D., Ron Vale, Ph.D..
Cellular and Molecular Pharmacology, University of California San Francisco, San Francisco, CA, USA.

Cytoplasmic dynein is a microtubule-based motor protein involved in various cellular processes such as organelle transport and cell division. Using optical trapping microscopy and single-molecule fluorescence microscopy, we have characterized the force-production and stepping behavior of yeast cytoplasmic dynein. In contrast to prior studies, we find that dynein's stepping behavior can be characterized by two distinct stepping-modes. In the first mode, referred to as "common-mode" or "C-mode", dynein takes several consecutive forward steps followed by a single or occasionally a few consecutive backward steps. At loads of 1 pN, C-mode stepping is characterized by a mixture of 8, 12, 16 and 20 nm steps with the 8 nm step being the predominant step size. At forces close to dynein's stall force of 7 pN, single dynein molecules advance with predominantly 8 nm steps and minor fractions of ~4 and 12 nm steps. Intriguingly, as load increases (3-6 pN), dynein exhibits frequent forward-backward stepping (referred to as "FB-mode" stepping) characterized by large force-dependent displacements (12-24 nm). To dissect the structural requirements for the observed stepping behavior, we have engineered artificially dimerized truncated motors in which the putative distance between both heads has been decreased by different amounts. Interestingly, the shortest dimer capable of processive movement shows both a decreased step size and frequency of FB-stepping as well as a reduced stall force (4 pN). Strikingly, stall force, step size and the probability for FB-stepping of the shortest dimer can all be partially rescued by inserting 12 nm-long spacer elements (alpha-actinin repeats) between the heads and the beginning of the dimerization domain. Our data support a model in which rearward loads and the reduction of the linker domain connecting the dynein heads impose constraints that reduce the reach of the motor during ATP-dependent stepping.

Coordination Between the Actin-Binding Cleft and Active Site of Smooth Muscle Myosin.

Christopher L. Berger, Ph.D.[1], Lynn R. Chrin, B.S.[1], Donald P. Gaffney II, B.S.[1], C. Ian Robertson, Ph.D.[1], Jared Cochran, Ph.D.[2], Susan P. Gilbert, Ph.D.[2].
[1]Molecular Physiology & Biophysics, University of Vermont, Burlington, VT, USA, [2]Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA.

Non-processive motor proteins such as myosin II appear to alternate between conformations in which they can bind either nucleotide or actin tightly, but not both. It has been suggested that the reciprocal nature of nucleotide- and actin-binding affinity is coordinated by movements of the upper 50 kD subdomain, in which closure of the actin-binding cleft opens the nucleotide-binding pocket and vice-a-versa. The former structural change is thought to facilitate actin-binding and product release, while the latter initiates nucleotide binding concomitant with dissociation from actin. To directly examine the dynamic relationship between the active site and actin-binding cleft during active cycling in solution, we have generated mutants of smooth muscle myosin containing spectroscopic probes in the active site, actin-binding cleft, or both. F425W in the actin-binding cleft of smooth muscle myosin shows nucleotide dependent changes that are directly correlated with actin binding/dissociation and opening/closing of the actin-binding cleft (Yengo et al., JBC 2002 277:24114). FRET between F344W and mant-nucleotides demonstrates nucleotide dependent changes associated with opening/closing of the active site (Robertson et al., Biophys. J. 2005 89:1882). However, the kinetics upon binding ATP are more complex for the F344W mutant than for the F425W mutant, showing an additional isomerization at the active site not seen in the actin-binding cleft. To further resolve this issue, a double mutant containing both F344W and F425W has been generated. Preliminary results support the findings from the single mutant studies, suggesting that structural changes at the active site and actin-binding cleft are not directly coupled through movement of the upper 50 kD subdomain.

Are the structures of F-actin and the F-actin backbone in the strongly bound actin-myosin Rigor complex different?

Rasmus R. Schroeder[1], Werner Jahn[2], Kenneth C. Holmes[2].
[1]Structural Biology, Max-Planck-Institute of Biophysics, 60438 Frankfurt am Main, Germany, [2]Max-Planck-Institute for Medical Research, 69120 Heidelberg, Germany.

The structure of actin monomers in F-actin filaments with and without strongly bound myosin is still not known in detail. To study the different structures we compared F-actin models derived from the cryo-EM 3D-reconstruction of the actin-myosin Rigor complex (Holmes et al., 2003; 1.3nm resolution) and X-ray diffraction patterns from oriented F-actin sols (Holmes et al., 1990; 0.7nm resolution). To obtain the F-actin density from that of the reconstructed actin-myosin complex we subtracted the density of myosin in its closed-50kDa-domain-cleft conformation. This provides the density for F-actin in the Rigor complex. New molecular F-actin models were then calculated in a least-squares structure refinement of the four actin monomer subdomains. In the refinement we optimized global orientation along the helical filament at 28/13 periodicity and relative orientation of subdomains within one actin monomer. As target functions we used a) the 3D-density of F-actin reconstructed from EM, b) the X-ray diffraction patterns, and c) constraints on the alpha-C backbone, which keep subdomain junctions at a correct distance (0.38nm). The target functions can be weighted during refinement, which allows us to calculate different models biased either to EM or diffraction data. Starting with a crystallographic G-actin structure (pdb entry 1G6Z) we find that the two models of F-actin (biased by EM or X-ray) are almost identical at a resolution level of about 1 nm. However, the EM biased model seems to be slightly more compact.
To verify the structures of F-actin on its own and in the actin-myosin complex at higher resolution we have started to collect data on a 200KeV FEG electron microscope with an in-column filter. Preliminary image processing shows an improved resolution when compared with previous data.

A detailed kinetic analysis of the cardiac myosin activator, CK-1213296, suggests it improves cardiac contractility by accelerating transition from the weak to strongly bound states.

James J. Hartman, **Ph.D**.[1], Hector M. Rodriguez, Ph.D.[1], Sheila Sylvester, B.S.[1], Y T. Shen, M.D.[2], David A. Kass, M.D.[3], Stevphen F. Vatner, M.D.[2], Fady Malik, M.D., Ph.D.[4], Roman Sakowicz, Ph.D.[1].
[1]Biochemistry, Cytokinetics, Inc., South San Francisco, CA, USA, [2]Department of Cell Biology & Molecular Medicine, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA, [3]Johns Hopkins, Baltimore, MD, USA, [4]Cytokinetics, Inc., South San Francisco, CA, USA.

We have identified a small molecule activator, CK-1213296, of the cardiac myosin ATPase. This agent was characterized in steady state and transient kinetic assays to understand its mechanism of action. CK-1213296 increases the steady-state rate of ATP hydrolysis of purified bovine cardiac myosin subfragment-1 (S1) and actin, as well as in bovine cardiac myofibrils, where the sarcomere structure is intact. We also find that CK-1213296 activates cardiac myosin selectively since no activation is observed in systems containing rabbit skeletal or chicken gizzard smooth muscle myosins. Analysis of the individual steps in the enzymatic cycle of cardiac myosin suggests that the actin stimulated release of phosphate is increased in the presence of CK-1213296. Additionally, we find that no other steps in the enzymatic cycle are affected by CK-1213296. Thus, the enzymatic step governing the weak to strong transition of S1 binding to actin is accelerated without affecting the release from the strongly bound state. This acceleration in the rate of transition from the weak to strong binding state appears to underlie its ability to increase force production in a dog model of cardiac function.

PHOSPHORYLATION OF THE MYOSIN REGULATORY LIGHT CHAIN AFFECTS THE CONFORMATION AND AFFINITY FOR ADP OF THE NUCLEOTIDE POCKET

Roger Cooke, PhD[1], Nariman Naber, PhD[1], Tomas Purcell, PhD[1], Edward Pate, PhD[2].
[1]biochem/Biophys, Univ california, San Francisco, CA, USA, [2]Applied Mathematics, Washington State Univ., Pullman, WA, USA.

A subunit of myosin, the regulatory light chain, becomes phosphorylated during muscle use and fatigue. We have previously found that myosin phosphorylation, low pH and high phosphate act synergistically to inhibit fiber velocity. Inhibition is observed at high temperatures, 30 C, but not at low temperatures. To investigate the mechanism of inhibition of velocity we have used spin-labeled ADP to monitor the conformation of the nucleotide pocket of fast skeletal myosin. Phosphorylation of the light chain in muscle fibers induced a slight opening of the nucleotide pocket. The affinity for spin-labeled ADP was also increased. In both of these observations the phosphorylated fast fibers resemble slow muscle fibers, which also bind ADP more tightly and have a more open nucleotide pocket than found in fast muscle fibers. These results suggest that the effect of myosin phosphorylation on fiber velocity is due to altered binding/release of nucleotides. The effects of myosin phosphorylation observed here could explain some of the inhibition in velocity seen in living fibers during fatigue

Large Conformational Changes in a Kinesin Motor Catalysed by Interaction with Microtubules

Keiko Hirose, Ph.D.[1], Toshihiko Akiba, Ph.D.[2], Sharyn A. Endow, Ph.D.[3], Linda A. Amos, Ph.D.[4].
[1]Research Institute for Cell Engineering, AIST, Tsukuba, Ibaraki, Japan, [2]Biological Information Research Center, AIST, Tsukuba, Ibaraki, Japan, [3]Department of Cell Biology, Duke University Medical Center, Durham, NC, USA, [4]MRC Laboratory of Molecular Biology, Cambridge, United Kingdom.

Kinesin motors release nucleotide upon interaction with microtubules, then bind and hydrolyse new ATP to move along the microtubule. Although crystal structures of kinesin motors with bound nucleotide have been solved, nucleotide-free structures have not yet been reported. To understand the structural changes in kinesin motors bound to microtubules, we studied the 3D structure of microtubules decorated with a Kinesin-14 motor, Kar3, without nucleotide, as well as with ADP and AMPPNP, using electron cryo-microscopy and computer 3D reconstruction. The resolution of the 3D maps we obtained is unusually high, ~10-12 Å, sufficient to show α-helices, e.g., tubulin helices H3, H11 and H12, and the motor Switch II helix, α4. Although differences between the ADP and AMPPNP states were relatively small, consistent with published X-ray crystallography data, we found large structural changes in the motor bound to microtubules without nucleotide, including melting of helix α4, closure of the nucleotide-binding pocket, and movement of loop L7 towards the microtubule. The movement of L7 was accompanied by distortion of the central β-sheet, reminiscent of nucleotide-free myosin crystal structures (Reubold et al., 2003; Coureux et al., 2003). Contrary to reports by others for the Kinesin-3 motor, Kif1A (Kikkawa et al., 2001), the Kar3 motor domain did not rotate between the motor-MT ADP and ATP-like states. We propose that the Switch II region of the motor controls docking of the Kar3 neck by conformational changes in the central β-sheet, similar to myosin, rather than by rotation of the motor domain, as proposed for Kif1A.

Dissection of Intra-molecular Communications between the Catalytic Head and Microtubule-binding Site in the Dynein Heavy Chain

Takahide Kon, **Ph.D.**, Kenji Imamula, Reiko Ohkura, Kazuo Sutoh, Ph.D..
Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Tokyo, Japan.

Communications between the track-binding site and ATPase site are essential for the motile activity of motor proteins. In dynein, its ATPase site(s) is located within the head domain containing six AAA+ modules, while the microtubule (MT)-binding site called stalk head is spatially separated from the head domain by a 10-15 nm slender stalk that is predicted to be formed from an antiparallel coiled coil. Therefore, the information at the ATPase site and MT-binding site of dynein must be transmitted through the long stalk coiled coil. However, the mechanism underlying this bidirectional communications remains unclear.
Recently, it has been proposed that shifts in the alignment of two helices forming the stalk coiled coil modulate the MT-binding affinity of the stalk head (Gibbons et.al., J. Biol. Chem. (2005) 280, 23960-23965). Here we employed a disulfide cross-linking strategy to elucidate the roles of the predicted shifts within the stalk coiled coil in the bidirectional communications. Our results showed that when the two helices were cross-linked by a disulfide bond, the dynein displayed MT-independent "activated" ATPase activity and trapped in an ATP-insensitive "strong-binding" state. Conversely, after another type of crosslinking that is expected to cause a small shift in the alignment of the coiled coil, the dynein exhibited MT-independent "basal" ATPase activity with an ATP-insensitive "weak-binding" state. These results strongly suggest that dynein utilizes small conformational changes within the stalk coiled coil to couple the MT-binding and ATPase activities.

Loading direction controls the ADP affinity of myosin V

Yusuke Oguchi[1], Sergey V. Mikhailenko[1], Adrian O. Olivares[2], Enrique M. De La Cruz[2], Shin'ichi Ishiwata[1].
[1]Dept.Phys, Waseda Univ, Tokyo, Japan, [2]Molecular Biophysics & Biochemistry Department, Yale University, New Haven, CT, USA.

Myosin V performs many consecutive 36 nm steps along an actin filament accompanied by a cyclic ATP hydrolysis, before it detaches from the filament. For such processivity to be realized, two identical heads of myosin V are considered to communicate with each other. Several recent studies have supported the idea that an internal strain exists between the two bound heads, which is a key factor regulating the nucleotide state of each bound head. However, these load-dependent states have not yet been clearly characterized. To demonstrate the existence of the load-dependent nucleotide state, we obtained the unbinding-force distribution for a single-headed myosin V (MV-S1-1IQ) bound to an actin filament in the rigor state (in the absence of nucleotides) and the ADP-bound state (in the presence of 1 mM ADP) by using optical tweezers apparatus similar to that used for measuring the unbinding -force distribution for a kinesin-microtubule complex (Kawaguchi, K. & Ishiwata, S., *Science* **291**, 667-9, 2001). The distributions at the intermediate ADP concentrations were bimodal, with the two peaks corresponding to the rigor and the ADP-bound states, and the relative population of the two states changed with [ADP]. From these experiments, we found that the ADP affinity of MV-S1-1IQ is different for the plus-end and the minus-end loading. In addition, the difference in ADP affinities under the plus-end loading and minus end loading became larger, when the load was applied by some angle to the long axis of the actin filament. These results strongly suggest that the off-axis strain is important for regulating the ADP affinity of native double-headed myosin V. Such asymmetrical affinities of ADP under the load are expected to be a basis for the effective processive movement of myosin V.

A Kinetic Study of the CENP-E ATPase

Marilyn van Duffelen, **Ph.D.**, Steven S. Rosenfeld, MD, PhD.
Neurology, Columbia University Medical Center, New York, NY, USA.

CENP-E is a mitotic kinesin involved in the movement of chromosomes to the mid-zone during mitosis. Little is known about its enzymology. Therefore we have undertaken a study to determine the kinetics of the CENP-E ATPase cycle. We have constructed an Xenopus CENP-E construct consisting of the motor domain and the neck-linker (residues 1-342). The microtubule-activated kcat of this ATPase was found to be 8.4 sec^{-1} with a Km of 0.26   M at 22°C. We have also begun to investigate the transient state kinetics of the motor ATPase cycle. The apparent second order rate constant of mantATP binding in the presence of microtubules is 3.1   M^{-1}s^{-1} at 20°C. Other steps in the cycle, including P*i* release and neck linker movement, are under investigation and will be discussed.

Defining Elements of Yeast Cytoplasmic Dynein Required for Motility

Andrew P. Carter, PhD, Samara L. Reck-Peterson, PhD, Ahmet Yildiz, PhD, Arne Gennerich, PhD, Ronald D. Vale, PhD.
Cellular and Molecular Pharmacology, UCSF, San Francisco, CA, USA.

Cytoplasmic dynein is a microtubule motor protein with many important functions in eukaryotic cells. The dynein heavy chain consists of an N-terminal tail domain responsible for cargo binding and dimerization and a C terminal motor core. The motor core is made up of a linker element and a ring of six AAA+ domains and binds microtubules via a ~10nm coiled coil stalk. In order to understand how dynein generates movement it would be helpful to address which elements of the motor core are required for motility.
To address this we have used S.cerevisiae as an expression system, which has the advantage that homologous recombination can be used to readily manipulate the genomic copy of cytoplasmic dynein. Starting with a 331kD fragment of yeast dynein (equivalent to the 380kD construct defined by labs working with dictyostellium and rat dynein) we made a series of truncations along the linker region towards the first AAA+ domain. We showed that we could generate a 314kD construct that had similar speed (80nm/s) in a gliding assay to both the 331kD fragment and full length yeast dynein. Further truncation, by as little as 26 amino acids, destroyed both gliding motility and ATPase activity. These data suggest that ~380 amino acid linker region in the 314kD construct interacts closely with the AAA+ domains in order to generate movement.
To further define whether this linker was solely responsible for dynein motility we generated a 314kD construct with GFP at the C terminus of the AAA+ core (instead of at the N-terminal end of the linker domain). Surprisingly we still saw slow (~3nm/s) movement with this construct suggesting that there are movements in the dynein head independent of the linker region that also contribute to dynein’s movement.

Actin nucleators working together - or - Spire keeps surprising us

Margot E. Quinlan[1], Eugen Kerkoff[2], R. Dyche Mullins[1]
[1]Dept. of Cellular and Molecular Pharmacology, University of California San Francisco
[2]BayGene and MSZ, Universität Würzburg, Germany

There are three known cellular factors responsible for constructing actin networks: the Arp2/3 complex, the formins and Spire (Spir). They have distinct nucleation mechanisms and are associated with different actin structures. Only Spir is restricted to metazoans, suggesting that it is specialized to build actin networks specific to multicellular organisms. Direct coordination between distinct actin nucleators has not been described before and begs the question of why cells require a complex that can nucleate by two mechanisms and bind microtubules. To address this question we have studied the individual components and their interactions.

Spir assembles actin nuclei using four monomer-binding WH2 domains and an additional motif (L3), conserved among Spir proteins. We find that each WH2 domain plays a distinct role in the nucleation reaction and that the identity and position of each WH2 domain is important for maximal activity. L3 contains about 15 amino acids. Despite its size, it plays an essential role in nucleation. In fact, using L3 we were able to build an artificial nucleator. When placed between the two WH2 domains from N-WASp, which normally inhibit spontaneous actin polymerization, L3 converts these actin binding domains into a nucleator.

Capu is a formin which lacks conserved autoinhibitory domains and whose mode of regulation is unknown. We find that Spir potently inhibits actin nucleation by Capu. In contrast, Capu enhances actin nucleation by Spir. Spir also inhibits Capu-microtubule binding. Spir binds directly to Capu with nanomolar affinity through the Spir-KIND (Kinase Non-catalytic C-lobe Domain) domain and the Capu-FH2 (Formin Homology-2) domain. The KIND-FH2 interaction is specific to Spir- and Cappuccino-family proteins and is conserved in mammals. We propose that the Spir and Capu collaborate to create an actin network and coordinate microtubules, thereby regulating cytoskeletal landmarks essential to polarity establishment in *Drosophila* oogenesis.

Session: Force-generating mechanisms

Myosin S1 structure revealed by multifrequency EPR and site-directed spin labeling

Yuri E. Nesmelov[1], Valentin V. Novikov[1,2], Adam Burr[1], Roman Agafonov[1], David D. Thomas[1].
[1]Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN, USA,
[2]Engelhardt Institute of Molecular Biology RAS, Moscow, Russian Federation.

We have used multifrequency EPR, with an iodoacetamide spin label attached to Cys 707, to analyze the structural dynamics of myosin S1 in the vicinity of the relay helix and the converter domain. EPR spectra of spin-labeled S1 in solution, acquired at 9.4 GHz and 94 GHz, were interpreted in terms of a model that includes two modes of rotational motion: rapid (subnanosecond) restricted rotation in a restoring potential, and global (100s of nanoseconds) isotropic tumbling of the whole protein. EPR spectra were simulated according to this motional model and fitted to experimental spectra. Local myosin structure in the vicinity of the probe was interpreted in terms of the spin label's equilibrium orientational distribution, as characterized by the rapid restricted motion. To compare EPR data with available crystal structures, MD simulations of spin labeled myosin motion were performed. The orientation of the spin label in the molecular frame of myosin was determined at each step in the calculated MD trajectory, and the resulting distribution of Euler angles was used to simulate the EPR spectrum, which was then compared with experimental EPR spectra. Data for myosin S1, as well as for myosin S1 trapped with nucleotide analog are discussed. This study establishes the basis for future analysis of the structural dynamics of myosin during the actomyosin ATPase cycle. [Supported by NIH Grant AR32961 to DDT, NIH Grants AR48961, AR53562 to YEN and by University of Minnesota Supercomputing Institute to YEN].

Structural modeling of the N-terminal domain of the essential myosin light chain and its functional analysis in the intact heart.

Ewald Mirko Aydt, PhD[1], Hannelore Haase, PhD[2], Gilla Tünnemann[2], Cristina Cardoso, PhD[2], Gerhard Wolff, PhD[1], **Ingo Morano**, **PhD**[2].
[1]Revotar Biopharmaceuticals, Hennigsdorf, Germany, [2]Molecular Muscle Physiology, Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany.

The 3D structure of Type-II myosin comprises the bell-shaped motor domain and the alpha-helical neck domain associated with an essential light chain (ELC) and a regulatory light chain (subfragment-1 (S1)). A large part of the N-terminus of the ELC (49 amino acids) is missing in this model which therefore resembles the spliced ELC isoform (A2)of fast-twitch skeletal muscle.The predominant myosin, however represents the S1 associated with the A1, i.e the full-length ELC. The N-terminus of A1 consists of a repetitive Ala-Pro rich elongated rod segment (residues 15-30) protruding a highly interactive element at the most N-terminus which binds to the C-terminal residues 360-364 of actin. We modelled the missing 49 N-terminal amino acid to the ELC of the contemporary 3D model of myosin-S1 in order to yield the S1(A1). The missing 49 amino acids revealed an elongated structure with a length of 9.1nm, i.e. long enough to bridge the gap between A2 and the corresponding ELC binding site on the actin filament. In addition, we investigated whether the expression of N-terminal A1 peptides could improve the intrinsic contractility of the heart. We, therefore generated transgenic rats (TGR) which overexpressed minigenes encoding the N-terminal 15 amino acids of human atrial A1 (TGR/hALC-1/1-15) or human ventricular A1 (TGR/hVLC-1/1-15) isoforms in cardiomyocytes. Corresponding synthetic N-terminal peptides revealed specific actin binding. Expression of N-terminal human MLC-1 peptides in TGR (range: 3-6μM) was associated with significant ($p<0.001$) improvements of the intrinsic contractile state of the isolated perfused heart. Using synthetic hVLC-1/1-15 as a TAT fusion peptide, we observed specific accumulation of the peptide at the sarcomere in intact adult cardiomyocytes as well as a dose-dependent increase of shortening amplitude at constant activating intracellular free Ca^{2+}.

Roles of multiple nucleotide-binding/hydrolysis sites of the cytoplasmic dynein motor domain in microtubule sliding

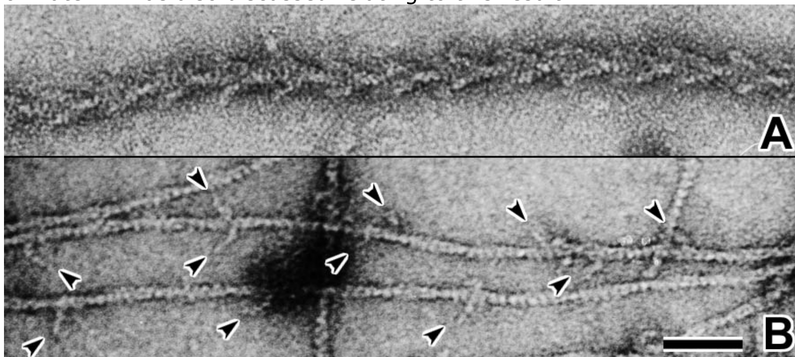
Kazuo Sutoh, Toshifumi Mogami, Kenji Imamula, Tomohiro Shima, Reiko Ohkura, Takahide Kon.
Dept Life Sciences, University of Tokyo, Tokyo, Japan.

Dynein is a member of the AAA+ family and has a characteristic ring-like motor domain. The AAA ring contains six AAA modules, among which four have putative nucleotide-binding/hydrolysis site. The question we are going to address is: what are physiological roles of these multiple nucleotide-binding/hydrolysis sites of cytoplasmic dynein in generating microtubule sliding? We introduced mutations into Walker A and Walker B motifs in the AAA modules of the Dictyostelium cytoplasmic dynein motor domain fused with GFP-based FRET sensors, and followed steady sate and transient kinetics of FRET changes and ON/OFF switching of microtubule-binding during ATP hydrolysis cycle. The results showed: (1) each of the AAA1, AAA3 and AAA4 modules of the motor domain has ATPase site, (2) ATPase cycles at these three sites are tightly coupled, and (3) the AAA1 module has the primary ATPase site responsible for the tail swing and for the on/off switching of microtubule-dynein binding, two steps essential for the microtubule sliding. The AAA2 module which lacks the consensus Walker B motif is likely to have a selective ADP-binding site, which may regulate the motile function of dynein as previously reported for axonemal dyneins.

Why can Acto-S1 chimera protein filaments move on a myosin-coated surface as fast as skeletal F-actin, and **why do water molecules around F-actin become more mobile** than bulk water upon interaction with S1?

Makoto Suzuki, **Professor**[1,2], Takashi Miyazaki[1,2], Md.Shahjahan Parvez Siddique, Dr.Eng.[1], Eisaku Katayama, Dr.Sci.[3], Taro Q.P. Uyeda, Dr.Sci.[4], George Mogami[1], Syed Rashel Kabir, Dr.Eng.[5], Tetsuichi Wazawa, Dr.Sci.[6].
[1]Materials Processing, Tohoku University, Sendai, Japan, [2]Life, CREST, JST, Sendai, Japan, [3]Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [4]Gene Function Research Center, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, [5]Tohoku University, Sendai, Japan, [6]Materials Processing, Tohoku University, CREST JST, Sendai, Japan.

Acto-S1 chimera proteins CP24 and CP18 carry the entire actin sequence, inserted in loop 2 of the motor domain of Dictyostelium myosin II, and have MgATPase activity close to that of natural Dictyostelium actomyosin. Here, we demonstrate sliding movement of homopolymer filaments (Fig.A) of CP24 or CP18 as fast as skeletal actin filamemts even though the subdomain 1 of actin part of the chimera protein is not exposed to external myosin coated on a glass surface. We detected cooperative structural change of actin filaments interacting with myosin S1using copolymer filaments (Fig.B) consisting of pyrene-labeled skeletal actin (SA) and either CP24 or CP18. Upon addition of ATP, the relative fluorescence intensity at 390 nm increased with the molar ratio of chimera to skeletal actin using 365 nm-excitation, demonstrating a sigmoid behavior. Stoichiometric analysis indicates that each CP24 molecule appears to affect four actin molecules, on average. Strong correlation between the fluorescence intensity and the rotational mobility of water will be also discussed relating to this result.



Mechanochemical coupling in myosin motor domain:
insights from combined potential of mean force,
molecular dynamics, normal mode and statistical coupling analysis

Qiang Cui, **Ph.D.**.
Chemistry, Univ. of Wisc., Madison, Madison, WI, USA.

Understanding the structure-function relationship in myosin, a characteristic molecular motor, relies on a quantitative description of how the local structural changes associated with ATP hydrolysis are propagated for a long distance to produce the dramatic swing of the lever arm and to regulate the actin binding activity. To obtain a clearer mechanistic picture for such ``mechanochemical coupling", various complementary simulation approaches are employed to probe different aspects of the functional dynamics in the myosin motor domain in the detached states. Potential of mean force and targeted molecular dynamics simulations are carried out to explore the thermodynamic and kinetic characteristics for the coupling between the open/close transition in the active site and rotation of the converter domain. Normal mode and statistical coupling analyses are used to identify residues that mediate the coupling between the active site, the converter domain and the actin-binding interface. Equilibrium molecular dynamics with both classical and hybrid quantum mechanical/molecular mechanical potentials are used to explore the effect of nucleotide chemical state (ATP vs. ADP-Pi) on the structural stability of the active site. Taken together, the results reveal a detailed description for how the thermodynamic and kinetic constraints required for the function of myosin are implemented in structural and energetic terms, which forms a solid basis for the development of coarse-grained models for analyzing the entire functional cycle of myosin. The computational strategy established in this study is applicable to the mechanistic analysis of many other molecular motor systems, for which revealing how collective conformational dynamics are modulated by local chemical events is the key challenge.

Temperature change does not alter single molecular force generated between the regulated actin filament and HMM

Masataka Kawai, **PhD**[1], Takanori Kido, MS[2], Martin Vogel, PhD[3], Rainer HA Fink, PhD[3], Shin'ichi Ishiwata, PhD[2].
[1]Anatomy and Cell Biology, University of Iowa, Iowa City, IA, USA, [2]Physics, Waseda University, Tokyo, Japan, [3]Medical Biophysics Unit, Institute of Physiology and Pathophysiology, Ruprecht-Karls-Universität, Heidelberg, Germany.

The temperature dependence of sliding velocity, force, and the number of cross-bridges was studied on regulated actin filaments (reconstituted thin filaments) when they were placed on heavy meromyosin (HMM) attached to a glass surface. The regulated actin filaments were used, because our previous study on muscle fibers demonstrated that the temperature effect was much reduced in the absence of regulatory proteins[4]. A fluorescently labeled thin filament was attached to the gelsolin-coated surface of a polystyrene bead. The bead was trapped by optical tweezers, and HMM-thin filament interaction was performed at 20-35°C to study the temperature dependence of force at the single molecule level. Our experiments showed that there was a small increase in force with temperature (Q_{10}=1.43) and sliding velocity (Q_{10}=1.46). The small increase in force was correlated with the small increase in the number of cross-bridges (Q_{10}=1.49), and when force was divided with the number of cross-bridges, the result did not depend on the temperature (Q_{10}=1.03). These results demonstrate that the force each cross-bridge generates is fixed and independent of temperature; the results are consistent with those on the microtubule-kinesin interaction[5] and unregulated actin filament-HMM interaction[6]. Our additional experiments demonstrate that tropomyosin (Tm) in the presence of troponin and Ca^{2+} enhances both force and velocity, and a truncated mutant Δ23Tm[7,8] diminishes force and velocity. These results are consistent with the hypothesis that Tm in the presence of Tn and Ca^{2+} applies positive allosteric effect on actin to make actomyosin linkage more secure so that larger force can be generated.

4. Fujita, Kawai. *JP* **539**,267-76 (2002)
5. Kawaguchi, Ishiwata. *BBRC* **272**,895-9 (2001)
6. Kawai, Kawaguchi, Saito, Ishiwata. *BJ* **78**,3112-9 (2000)
7. Hitchcock-DeGregori, Varnell. *JMB* **214**,885-96 (1990)
8. Landis, Back, Homsher, Tobacman. *JBC* **274**,31279-85 (1999)

Mechanistic consequences of movement on two-state tension generation in skeletal muscle

Julien S. Davis, Neal D. Epstein.
Molecular Cardiology, NIH, Bethesda, MD, USA.

Tension generation in muscle occurs during the attached phase of the ATP powered cyclic interaction of myosin heads on thick filaments with actin binding sites on thin filaments. It can be studied by the application of small step-perturbations to maximally Ca^{++} activated muscle fibers contracting under isometric conditions. Here, we contrast the remarkably simple, but different pathways of tension generation recorded in laser temperature-jump (T-jump) and classical length-jump (L-Jump) experiments. We found that the kinetics of tension generation following a T-jump fit a simple two-state reaction scheme. This enabled the forward (k_1) and reverse (k_{-1}) rate constants for tension generation to be determined for the first time. Increasing temperature accelerates k_1 and slows k_{-1}. Movement of thick and thin filaments is minimal in these experiments. Consequences of movement are probed in L-jump experiments. Apart from an acceleration of rate from the step-release, the T- and L-jump kinetics are virtually indistinguishable. Reaction amplitude vs. isometric tension plots are, however, entirely different: The T-jump plot is bell shaped and thus two-state in form. The L-jump plot is linear. Each, places tight constraints on mechanism: Following a T-jump, pre-force generating and force generating states simply re-equilibrate to generate a higher tension in apparent isolation. Following an L-jump step-release, tension recovery appears mediated by a group of nontension-generating cross-bridges rapidly triggered to generate tension by movement. The mechanism serves to buffer muscle tension against small changes in length. Arrhenius/anti-Arrhenius behavior, in which the forward rate constant increases while the reverse rate constant slows with increasing temperature, is seen in both types of experiment and is typical of the folding/unfolding kinetics of small proteins. We surmise that localized unfolding of the actomyosin cross-bridge creates the power stroke state.

Identifying the Power Stroke Step of Myosin V Using Computational Dwell-Time Distribution Analysis

Jung-Chi Liao, **PhD**[1], David Parker[2], Thomas J. Purcell, PhD[3], James A. Spudich, PhD[4], Scott L. Delp, PhD[1].
[1]Bioengineering, Stanford University, Stanford, CA, USA, [2]Mechanical Engineering, Stanford University, Stanford, CA, USA, [3]Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA, USA, [4]Biochemistry, Stanford University, Stanford, CA, USA.

The sequence of the mechanochemical steps involving the power stroke and the Pi release remains an open question for myosin. It is difficult to resolve this issue because the power stroke step and the Pi release step occur so rapidly that it is difficult to determine if they are separate steps, and if so, which one occurs first. Laser trap assays, however, can accelerate or decelerate the power stroke step so that the kinetic flux is redistributed, making it possible to distinguish these steps. We have developed a novel computational algorithm that can predict dwell-time distributions of any complex kinetics and conduct global fitting for dwell-time distributions under various conditions. Using this new algorithm, we simulated the possible mechanochemical mechanism of one head of myosin V. By fitting the data of dwell-time distributions under different applied forces and nucleotide concentrations, we conclude that the power stroke is neither coupled to the Pi release step nor the ADP release step. Our analysis suggests that the power stroke happens after the Pi release step and before the ADP release step.

Dynamic polymorphism of single actin molecules in the actin filament

Hiroaki Yokota[1], Jun Kozuka[2,3], Yoshiyuki Arai[2,3], Yoshiharu Ishii[2], Toshio Yanagida[2,4].
[1]Department of Molecular Physiology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, [2]Formation of Soft Nanomachines, Core Research for Evolution Science and Technology, Japan Science and Technology Agency, Suita, Osaka, Japan, [3]Department of Biophysical Engineering, Osaka University, Suita, Osaka, Japan, [4]Department of Biophysical Engineering & Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka, Japan.

Actin filament dynamics are critical in cell motility. The structure of actin filament changes spontaneously and can also be regulated by actin-binding proteins, allowing actin to readily function in response to external stimuli. The interaction with the motor protein myosin changes the dynamic nature of actin filaments. However, the molecular bases for the dynamic processes of actin filaments are not well understood. Here, we observed the dynamics of rabbit skeletal-muscle actin conformation by monitoring individual molecules in the actin filaments using single-molecule fluorescence resonance energy transfer (FRET) imaging with total internal reflection fluorescence microscopy (TIRFM). The time trajectories of FRET show that actin switches between low- and high-FRET efficiency states on a timescale of seconds. If actin filaments are chemically cross-linked, a state that inhibits myosin motility, the equilibrium shifts to the low-FRET conformation, whereas when the actin filament is interacting with myosin, the high-FRET conformation is favored. This dynamic equilibrium suggests that actin can switch between active and inactive conformations in response to external signals.
Nat. Chem. Biol. **2**, 83-86 (2006)

Development of a high-pressure microscope and its application to kinesin-microtubule complex

Masayoshi Nishiyama, Ph.D[1], Yoshifumi Kimura, Ph. D[2], Yoshio Nishiyama[1], Masahide Terazima, Ph.D[1].
[1]Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto, Japan, [2]International Innovation center, Kyoto University, Kyoto, Japan.

Hydration, i.e., the intermolecular interaction between the protein and water molecule, is a very important factor for the structural formation of proteins and the enzyme activity. For studying this factor, it is desirable to modulate the interaction and monitor the structures or biological activity. For doing this, using a fact that the hydration effect could be modulated reversibly by applying the hydrostatic pressure, we have developed a high-pressure microscope that enables to monitor the protein structure and function at various hydrostatic pressures. This high-pressure cell can be used up to 200 MPa (~2000 bar) and it was mounted on a commercial inverted microscope to observe epi-fluorescent images of the individual microtubules tethered to kinesin molecules on the optical window of the high-pressure cell. Here we report two experiments using this cell. First, we observed the effect of pressure on the structure of microtubule. It was found that the microtubule depolymerized from the both ends even in the presence of 10mM Paclitaxel with increasing the pressure. The shortening velocity increased exponentially with the increment of pressure and it reached to ~6μm/min at 200 MPa. In addition, the breakage of microtubules was also observed at pressure levels of >150MPa. Second, we performed in Vitro motility assays in the presence of ATP molecules. The sliding velocity under the high pressures followed a Michaelis-Menten kinetics, in which both of the maximum velocity and the Michaelis constant decreased with the increment of the pressure. As the pressure increased, the velocity decreased linearly and reached to the half of its maximum at 150 MPa. We consider that these structural and functional perturbations are casued by penetration of water molecules into the intermolecular binding sites.

Step Size of Kinesin Measured by Electron Paramagnetic Resonance

Kazunori Sugata[1], Likai Song[2], Motoyoshi Nakamura[1], Shoji Ueki[1], Piotr G. Fajer[2], **Toshiaki Arata**[1].
[1]Dept Biol Sci, Osaka University, Toyonaka, Osaka, Japan, [2]Dept Biol Sci and NHMFL, Florida State University, Tallahassee, FL, USA.

By dipolar continuous-wave and pulse EPR methods we have determined a wide-ranged distribution of the distance between two spin labels placed at each of the neck-linkers of dimeric kinesin. In the absence of microtubules the distance represented a broad distribution centered at 3.6 nm with a width of ±1.0 nm, indicating that the linkers are random coils and extended as twice as expected from a crystal structure. In the presence of microtubules narrower distributions with a 0.2-0.3-nm width were recovered in the range of 1.5-2.5 nm as well as those at 3.6 nm. In the presence of ATP-analogs, AMPPNP and ATPγS, the 3.6-nm population was reduced remarkably and the shorter distribution at 2.0 nm was dominated, indicating that the linkers reduce the flexibility and retract to a half. EPR for the first time detected directly a length change of the specific peptide of motor protein on the nanometre scale. We propose that large nucleotide-dependent length and flexibility changes of the linkers contribute to the force- or bias-generation during the 8-nm stepping by a kinesin molecule along a microtubule.

Simultaneous observation of rotatory and stepping motions of single myosin II molecules

So Nishikawa[1], Toshio Yanagida[2].
[1]JST, CREST, Osaka, Japan, [2]Osaka university, Osaka, Japan.

Myosin moves on an actin filament which has a helical structure. Therefore, as it advances, myosin's heads and/or its lever arm are expected to rotate around the actin filament. The mechanism of the movement has been fairly well elucidated using a traditional laser trap system. However, at least in the case of non-processive myosin, conventional measurement systems can only reveal the information about the displacement along the filament's axis. This is one of the reasons why the difference in stepping movement between myosin II HMM (double headed) and S1 (single headed) has not been detected using conventional methods, and the necessity for myosin forming double heads in muscle is not clear.
In order to track the 3D motion of myosin II, we designed a novel experimental arrangement, in which azimuthal and lateral movement of the actin filament caused by the motion of a single myosin molecule can be measured simultaneously.
In brief, by using the multiplexed optical tweezers apparatus, an additional bead is attached to the middle of the actin filament to monitor the rotational motion.
To test the performance of this new measurement system, we measured the torsional rigidity of the filament under well-controlled tension (~1.5 pN). By studying the distribution of rotational angles as a function of the effective filament's length, we calculated the rotation rigidity to be 8 $\times 10^4$ pN.nm^2. This number is consistent with the previous report (ref. Yasuda *et al*, JMB, 1996) and ensures the precise measurement of the rotational angle.

A New Structural Model Reveals Energy Transduction in Dynein

Adrian W. R. Serohijos[1], Yiwen Chen[1], Feng Ding, PhD[2], Timothy C. Elston, PhD[3], Nikolay V. Dokholyan, PhD[2].
[1]Physics Department and Program in Molecular and Cellular Biophysics, University of North Carolina-Chapel Hill, Chapel HIll, NC, USA, [2]Department of Biochemistry and Biophysics, University of North Carolina-Chapel Hill, Chapel HIll, NC, USA, [3]Department of Pharmacology, University of North Carolina-Chapel Hill, Chapel HIll, NC, USA.

The microctubule-based dynein motor is involved in the transport of organelles and vesicles, the maintenance of the Golgi, mitosis, and the beating of cilia and flagella. However, unlike kinesin and myosin, the mechanism by which dynein converts chemical energy into mechanical force remains largely a mystery, which is primarily due to the lack of a high-resolution molecular structure. Here, using homology modeling and normal mode analysis, we propose a complete atomic structure and a novel mechanism for force generation by the motor protein dynein. In agreement with recent 3D electron microscopy (EM) density reconstructions showing dynein as a ring-shaped heptamer, our model consists of six ATPases of the AAA (ATPases associated with various cellular activities) superfamily and a C-domain, whose structure is unknown but is biochemically shown to control motor function. Moreover, the model shows a coiled-coil spanning the diameter of the motor which accounts for previously unidentified structures in EM studies. Furthermore, analysis of the most accessible large scale motions of the motor show that half of the motor is very mobile while the other half, which contains the nucleotide binding sites, remains rigid. Our analysis also demonstrates the likely domain rearrangements in the dynein motor unit that generate its power stroke. Our results provide new insights into the structure and function of dynein that can guide further experimental investigations on this fundamental motor protein.

Conformational Dynamics of the Nucleotide Binding Site in Fast and Slow Muscle Fibers

Nariman Naber, **Ph.D**[1], Tom Purcell, Ph.D[2], Edward Pate, Ph.D[3], Roger Cooke, Ph.D[4].
[1]Biochem, UCSF, San francisco, CA, USA, [2]CVRI, UCSF, San francisco, CA, USA, [3]Math, Washington State University, Pullman, WA, USA, [4]Biochem and Biophysics and CVRI, UCSF, San francisco, CA, USA.

We have used spin-labeled nucleotides to monitor the conformation of the nucleotide pocket of fast and slow muscle fibers and myosin subfragments. Our previous results have shown that the nucleotide pocket of fast skeletal myosin-ADP is closed and that it opens upon binding to actin. We extended our studies using S1 and HMM bound to Actin as well as muscle fibers. S1 was prepared from both slow and fast rabbit muscle. HMM was prepared from fast skeletal muscles. We used the 2',3'-SLATP analog for these studies. Our results show that the nucleotide pocket is in equilibrium between opened and closed states when S1 from both types of muscle is bound to actin. However the nucleotide pocket is more in the closed state in slow S1•Actin than in fast S1•Actin. The opposite result was found when the nucleotide is bound to muscle fibers. In muscle fibers the nucleotide pocket is more closed in the fast fibers and more open in the slow fibers. This result could be due to the fact that in the cross-bridges in fibers are two headed or to other interactions present in the fibers. When the nucleotide was bound to fast HMM•Actin , the nucleotide pocket was closed, similar to the result with fast fibers. This indicates that the interactions between the two heads of myosin influence the opening and closing of the nucleotide pocket.

The role of strong actin-myosin binding in muscle force and motion generation.

Josh E. Baker, PhD.
Biochemistry, University Nevada Reno, Reno, NV, USA.

Single molecule studies demonstrate that the actin-myosin strong binding transition is a critical mechanical step in the ATPase reaction; however, the relationship between the discrete, mechanical events of single myosin molecules and the steady state mechanochemistry of muscle remains unclear. We have studied the relationship between strong actin-myosin binding, muscle force generation, and in vitro actin sliding velocities. In a series of in vitro motility experiments, we studied the effects of different combinations of [ATP], [ADP], [Pi], temperature and NEM-myosin on actin sliding velocities. We showed that below a critical ATP concentration of approximately 100 μM, actin filament sliding is driven by an internal force collectively generated by myosin heads against the viscous damping of myosin heads strongly-bound to actin. Above the critical ATP concentration, actin sliding is significantly influenced by actin-myosin strong binding kinetics. These results challenge the conventional assumption that actin-myosin detachment kinetics is the sole biochemical determinant of actin sliding velocities. To study the biochemistry of force generation, we used electron paramagnetic resonance, sedimentation binding, and stopped flow kinetics of actin and myosin S1 binding in the presence of MgADP. We show that, as previously proposed, changes in the free energy for actin-myosin strong binding associated with changes in vanadate concentration and temperature correlate with changes in the average force per bound crossbridge measured in active isometric muscle. These data support a macroscopic model of muscle contraction similar to the one proposed by A.V. Hill in which i) muscle force is an emergent property of the muscle system collectively generated by many myosin molecules, and ii) muscle shortening is driven by this ensemble internal force against the viscous load of strongly-bound myosin heads.

Nucleotide Pocket Conformation Dynamics In Cardiac Muscle Myosin

Thomas J. Purcell, Ph.D.[1], Nariman Naber, Ph.D.[1], Kathy Franks-Skiba[1], Edward Pate, Ph.D.[2], Roger Cooke, Ph.D.[1].
[1]Biochemistry, University of California, San Francisco, San Francisco, CA, USA, [2]Math, Washington State University, Pullman, WA, USA.

We have extended our spin-labeled nucleotides studies to cardiac myosin. Mammalian cardiac myosin heavy chain (MHC) has two major isoforms, faster alpha-MHC which predominates in the atria and slower, more efficient beta-MHC which is found mainly in the ventricles. Injury and other environmental conditions influence the heavy chain distribution in the heart, suggesting that biophysical differences between the two isoforms play an important role in efficient operation of the heart. Initial studies with myofibrils isolated from pig atria and ventricles show similar results to fast and slow skeletal fibers. Both muscle preps showed a distribution of nucleotide pocket conformations, in the faster alpha-MHC dominated atrial muscle closed nucleotide pocket dominates the equilibrium, whereas the slower beta-MHC dominated ventricle muscle appears to have a more open nucleotide pocket. We are continuing the studies with purified myosin and actomyosin to further characterize the thermodynamic and conformational differences between the two cardiac heavy chains.

MECHANICS AND STRUCTURAL DYNAMICS OF MUSCLE MYOSIN UNDERLYING THE FORCE-VELOCITY RELATION.

G. Piazzesi[1], M. Linari[1], M. Reconditi[1], P. Bianco[1], E. Brunello[1], A. Stewart[2], T. Irving[3], D. Gore[3], M. Irving[4] and V. Lombardi[1].
[1]Laboratorio di Fisiologia, DBAG, Università di Firenze, Italy; [2]Dexela Ltd., London UK; [3]BioCAT, Advanced Photon Source, Argonne, USA; [4]Randall Division of Cell and Molecular Biophysics, King's College London UK.

The bipolar arrangement of myosin motors in the two halves of each myosin filament produces an X-ray interference effect that allows the axial motions of the motors to be followed in an intact muscle fiber with a precision of about 1Å. We use this effect in combination with fast sarcomere level mechanics to measure the working stroke of myosin motors *in situ*, as they pull the actin filaments towards the centre of the myosin filament during muscle shortening. Using the interference effect we demonstrated that the early phase of isotonic shortening following a step reduction in force from the steady value developed during the isometric contraction (T_0) is accompanied by a synchronised working stroke in the attached myosin heads that is ~6 nm at high load and becomes larger and faster reducing the load (Reconditi *et al*. *Nature* **428**, 578, 2004). Here we report combined measurements of both stiffness, total intensity and interference fine structure of the M3 reflection during steady shortening under different loads (0.9–0.15 T_0). The results indicate that in the region of high and intermediate loads (>0.4 T_0) the reduction of the load is accompanied by the reduction in the number of motors attached to actin, without substantial changes in the motor force and stroke size. These results provide a molecular explanation to the ability of muscle to generate work and power at high efficiency.

Supported by MUR, Italy; NIH (R01AR049033.03); MRC, UK.

Force generation mechanism of axonemal dynein inspired by the structure of isolated and *in situ* dyneins of *Chlamydomonas* axonemes

Kazuhiro Oiwa, **Prof**.[1,2], Hitoshi Sakakibara, Ph.D.[1], Takashi Ishikawa, Ph.D.[3].
[1]Kobe Advanced ICT Research Center, Kobe, Japan, [2]Graduate School of Life Science, University of Hyogo, Harima, Japan, [3]Department of Biology, Swiss Federal Institute of Technology, Zürich, Switzerland.

Eukaryotic cilia and flagella share a common “9+2” structure, in which nine peripheral doublet microtubules surround two central singlet microtubules. Outer and inner dynein arms projecting from each peripheral doublets are capable to extend to the neighboring doublet and slide it in one direction coupled with ATP hydrolysis. Dynein arms consist of one to three dynein heavy chains, together with many light chains and intermediate chains. We revealed the hexameric ring structure of axonemal dynein of *Chlamydomonas* and proposed the model in which coordinated multiple conformational changes amplify small conformational changes between 1st AAA domain and its neighbors by docking of the head ring onto a linker which connects the tail and the head ring. The resultant rotation of the head ring swings the stalk. Despite the increase in knowledge about isolated dyneins, much remains to be learned about the mechanisms underlying flagellar motion. To elucidate the role of dyneins in flagellar motility and the force generating mechanism of dynein *in situ*, we have carried out the electron tomography and reconstructed the 3D structure of *in situ* axonemal outer dynein arms in *Chlamydomonas* flagella in the absence of nucleotides. In our reconstitution of the wild-type outer dynein arm, it consists of three plates stacked up in parallel to the longitudinal axis of the A-subfiber and a tilted rod connecting these plates. The ring-like morphology found in the plates suggests that they correspond to the dynein head rings. The significant electron densities were also found connecting the rod and an adjacent outer dynein arm. These architectures suggests the mechanism to convert the power stroke of individual dynein molecules into the coordinated action of dynein arms for bend formation and propagation.

EMERGENT PROPERTIES OF ACTIN ORGANIZATION FROM STOCHASTIC SIMULATION OF THE DENDRITIC NUCLEATION MODEL

Edwin Taylor.
University of Chicago and Northwestern Univ., Chicago, IL, USA.

Emergent Properties of Actin Organization from Stochastic Simulation of the Dendritic Nucleation Model
Membrane protrusion is believed to be generated by dendritic nucleation, the branching polymerization of actin filaments by activated arp2,3 modulated by capping of filaments, debranching and depolymerizaton by cofilin. Wh;ile other factors may contribute this set of reactions is sufficient to produce motility in reconstituted systems. We have developed a computer model to simulate the dynamics of this system using, in so far as possible, known rate constants and the elastic properties of the filaments and the membrane. For each time step the change in elastic free energy of flexible actin filaments, bending free energy of membrane and surface free energy was calculated for addition of a subunit to the filament set to determine the Boltzman term. Monte Carlo method was used to determine occurance of this step. Important feature of flexible filament-flexible membrane model is that it leads to co-operativity , which reduces the size of thermal fluctuations needed to add a sub unit.
The main conclusion of the simulation is that that the dynamics of the system by itself is sufficient to produce a stable filament number and orientation distribution starting from an initial number and random orientation of the filaments. Examples will be given showing the evolution of the distribution by reproduction (branching) and selection for survival (Fisherian fitness). For a 70 degree branch angle the stable distribution is plus or minus 35 degrees from the perpendicular to the membrane. Time to reach the steady state distribution is shorter for this branch angle than for larger or smaller angles. The robustness of the distribution has been investigated for a broad range of physical and kinetic properties.

ATPase Mechanism of Eg5 in the Absence of Microtubules

Jared C. Cochran, Ph.D.[1], Susan P. Gilbert, Ph.D.[2].
[1]Chemistry, Dartmouth College, Hanover, NH, USA, [2]Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA.

The ATPase mechanism of kinesin superfamily members in the absence of microtubules remains largely uncharacterized. We have adopted a strategy to purify monomeric human Eg5 (HsKSP/Kinesin-5) in the nucleotide-free state (apoEg5) in order to perform a detailed transient state kinetic analysis. We have used steady-state and presteady-state kinetics to define the minimal ATPase mechanism for apoEg5 in the absence and presence of the Eg5-specific inhibitor, monastrol. ATP and ADP binding both occur via a two-step process with the isomerization of the collision complex limiting each forward reaction. ATP hydrolysis and phosphate product release are rapid steps in the mechanism, and the observed rate of these steps is limited by the relatively slow isomerization of the Eg5-ATP collision complex. A conformational change coupled to ADP release is the rate-limiting step in the pathway. We propose that the microtubule amplifies and accelerates the structural transitions needed to form the ATP hydrolysis competent state and for rapid ADP release, thus stimulating ATP turnover and increasing enzymatic efficiency. Monastrol appears to bind weakly to the Eg5-ATP collision complex, but after tight ATP binding, the affinity for monastrol increases, thus inhibiting the conformational change required for ADP product release. Taken together, we hypothesize that loop L5 of Eg5 undergoes an "open" to "closed" structural transition that correlates with the rearrangements of the switch-1 and switch-2 regions at the active site during the ATPase cycle.

Structure of the Microtubule-Kinetochore Interface Visualized by EM Tomography

Richard McIntosh, **Ph.D.**.
Mol. Cell and Devel. Biology, University of Colorado, Boulder, CO, USA.

Mitotic chromosome segregation requires MTs attached to kinetochores. Initial kinetochore attachment is to the lateral MT surface and produce minus end-directed forces. Gradually, kinetochores form more stable connections with MT plus ends, and thereafter chromosome motion is coupled to MT polymerization. Spindles contain many MT-dependent motors, but most are not essential for chromosome motion; they contribute only to the accuracy of mitosis. This motivates a study of the mechanically significant structures that couple MTs to kinetochores. Recent work shows that the DAM/DASH complex from budding yeast can form rings around MTs in vitro. These rings can follow the depolymerizing ends of a MT in vitro, and since some of their components associate with centromeric chromatin, there is enthusiasm for a MT-dependent, disassembly-driven machine that does not depend on familiar motor enzymes.
We have examined the structures of kinetochore-MT linkages in mammalian cells and both budding and fission yeasts, using cells that were cryo-immobilized in mitosis then fixed and embedded for electron microscopy by freeze-substitution. Electron tomography has been used to visualize the 3-D structure of the MT-kinetochore interface at different times in mitosis. Even in budding yeasts, no rings are evident, and deletion analysis shows that the DAM complex is not essential in fission yeast. The tomograms of all three species reveal slender fibrils that have one end on a strand of chromatin and the other associated with a MT, either its inner or outer surface or the tips of its splaying protofilaments. We propose that these fibrils are a mechanically significant component of the kinetochore-MT connection; through labile MT associations, they can track the disassembling end of the polymer, transducing the energy stored in the MT lattice into mechanical work to move the chromosome.

The Role of Fluid Surface Energy in Muscle Contraction and Motility - Was Szent-Györgyi Right After all?

Alex Metherell, PhD, MD.
Donald Bren School of Information & Computer Science, University of California Irvine, Irvine, CA, USA.

One cubic centimeter of muscle tissue contains a fluid filament surface area about the size of a tennis court. Yet little attention has been given to myoplasmic fluid surface energy (tension) in motility.
Albert Szent-Györgyi recognized in 1940-43 that contraction is accompanied by a dramatic change in the surface physics at the fluid filament boundary. At a 1970 symposium he said *"the greatest experience of my scientific life"* was the moment I saw " ...*motion produced for the first time in vitro by constituents of muscle* ..." He had earlier succeeded in extracting actomyosin which "... *forms a resilient gel. It is excessively hydrophillic."* "...*Under influence of ATP actomyosin loses hydrophillity. The hydrophillic colloid becomes entirely hydrophobic, the most striking change I ever saw."* He abandoned muscle work when he was unable to reconcile this finding with the sliding filament theory.
During contraction the filament chemical structure remains relatively unchanged, but the chemistry of the myoplasm changes dramatically. This can be expected to change the fluid surface energy resulting in the fluid going from proteophillic to proteophobic - explaining Szent-Györgyi's observations.
This paper considers the possible role of fluid surface energy in contraction. This may account for force generation, filament sliding, filament shortening, contraction beyond overlap and heat of shortening. The filaments define the fluid/filament boundary. The cross-bridges participate in the hydrolysis of the ATP changing the chemistry of the fluid thus changing the surface energy. A surface energy gradient is set up between the overlap and non-overlap region due to the thin film effect in the overlap region which may explain, in part, force and movement.
A meaningful theoretical model must be testable. This paper suggests experiments to confirm this hypothesis.

Functional Mechanism of Eg5, a Tetrameric Kinesin.

Christoph F. Schmidt[1], Mikhail J. Korneev[2], Stefan LakÃ¤mper[1], Benjamin H. Kwok[3], Tarun M. Kapoor[4].
[1]FakultÃ¤t fÃ¼r Physik, Georg-August-UniversitÃ¤t, GÃ¶ttingen, Germany, [2]Vrije Universiteit Amsterdam, Amsterdam, The Netherlands, [3]FakultÃ¤t fÃ¼r Physik, Rockefeller University, New York, NY, USA, [4]Rockefeller University, New York, NY, USA.

We have investigated the tetrameric kinesin motor Eg 5 both in single-molecule fluorescence assays and in single molecule laser trapping assays. Eg 5 is a processive kinesin and moves in 8nm steps, but the full length motor appears to behave quite differently from kinesin 1 in terms of its load dependence. We hypothesize that load-dependent release at relatively low forces might be relevant as a force-sensing device in the mitotic spindle.

Probe conformational transitions and dynamical couplings with normal modes of coarse-grained models

Wenjun Zheng, **PhD**, Bernard R. Brooks.
NIH, Bethesda, MD, USA.

Dynamics/motions are essential links between functions and structures of biomolecular complexes. However many biologically interesting dynamics are inaccessible to all-atom molecular dynamics (MD) simulations. Recently, an elastic network model (ENM) has been proposed to model the inter-atomic bonds as springs and the protein as a network of coupled harmonic oscillators. The normal modes analysis of the ENM was shown to give a handful of low-frequency normal modes that are capable of describing many crystallographically observed protein conformational changes. Based on ENM, we propose the following deformation analysis: we introduce a structural perturbation at the functional site (such as ATP binding site of myosin) that mimics the effect of ligand-binding, and then we compute the responding conformational changes of the whole structure to explore the large-scale collective motions trigged by ligand binding. This method is applied to the study of signal transduction in myosin motor from its ATP binding site to the mechanic elements where forces and motions are generated.
A new web server (http://enm.lobos.nih.gov) has been set up for public access to the above analysis.

Session: Motor walking mechanisms

Mechanism of an Unconventional Kinesin-3 Motor

Guenther Woehlke, Sarah Adio, Bettina Ebbing.
Adolf-Butenandt-Inst. Cell Biology, Munich, Germany.

Kinesin-3 motors show a complex dimerization behaviour that leads to unique motor properties. We show that the Kinesin-3 member NcKin3 from Neurospora crassa is a stable dimeric motor, unlike KIF1A or Unc104. It moves microtubules in a density-dependent fashion at a maximal velocity of 0.64 μm/s. ATPase, mant-ADP release and microtubule detachment characteristics show that the release of ADP from head 2 is not triggered by ATP in the microtubule-bound head, and that the motor catalyzes only one or few ATPase cycles upon a productive microtubule encounter. The duty ratio of the motor inferred from KM,ATP in motility and ATPase assays is below 10%, incompatible with a hand-over-hand model of motility. Together the data suggest that NcKin3 neither shares the dimerization peculiarities of other Kinesin-3 motors, nor does it move processively as Kinesin-1 motors, and thus functions according to a so far unknown mechanism.

INTERACTION BETWEEN MOTOR HEADS STRONGLY EFFECTS THE DYNAMICS AND BIOPHYSICAL PROPERTIES OF MOTOR PROTEINS

Anatoly B. Kolomeisky, Rahul Das.
Chemistry, Rice University, Houston, TX, USA.

Many motor proteins have two or more active heads that play a crucial role in the overall dynamics. Experiments show that most motor proteins with two motor domains move using the so-called hand-over-hand mechanism when the motor heads alternate their motion, although in the coordinated fashion. However, the mechanism of such coordination remains unclear. We present a simple stochastic model that proposes a possible mechanism of interaction between motor domains. The exact analytical solutions for biophysical properties of motor proteins are presented. It is shown that the interaction between the motor heads have a very strong effect on dynamics of motor proteins. Our theoretical approach suggests several ways of controlling and changing the properties of motor proteins that can be tested in single-molecule experiments.

Mechanism and Energy Coupling of Kinesin ATPases

David D. Hackney.
Department of Biological Sciences, Carnegie Mellon Univ., Pittsburgh, PA, USA.

Although much is know of the mechanism of hydrolysis of ATP by kinesins and how it is coupled to the free energy changes that result in movement, a number of key features are still not well established. Information about the reverse reactions can be obtained by analysis of the rate of exchange between oxygen atoms of water and phosphate that occurs as a consequence of hydrolysis of ATP. Recent application of this approach (Proc.Natl.Acad.Sci. 102, 18338 (2005)) has provided the rate for synthesis of bound ATP via addition of Pi to the microtubule complex of kinesin with ADP. Completion of the free energy profile for kinesin ATPase will also require information about the reverse rates in the absence of microtubules and analysis by the oxygen exchange technique is in progress. Studies of the rate of binding and release of mant-AMP-PNP from kinesin and the kinesin-microtubule complex indicate that AMP-PNP binds to the free enzyme significantly more tightly than to the microtubule complex. Because the steps of AMP-PNP binding and binding of kinesin to the microtubule are linked in a thermodynamic cycle, this indicates that free kinesin binds significantly more tightly to microtubules than does the complex of kinesin and AMP-PNP. This is not, however, necessarily also true of the complex with true ATP. There is evidence that kinesin exists in multiple conformational states that only slowly interconvert under some circumstances. With conventional *Drosophila* kinesin-1 in the absence of bound nucleotide, the enzyme is predominately in a ‘closed’ state that cannot rapidly rebind nucleotide (J.Biol.Chem. 269, 16493 (1994)), but which can slowly reactivate. Recent work indicates that similar equilibria exist for other nucleotide states and that the relative populations of the states and rates of interconversion differ considerably between superfamily members.

Single Molecule Observations of Structural Changes in the Neck Linker of Kinesin

Michio Tomishige[1], Ronald D. Vale[2].
[1]Department of Applied Physics, University of Tokyo, Tokyo, Japan, [2]Department of Molecular and Cellular Pharmacology, University of California, San Francisco, San Fransicso, CA, USA.

Kinesin moves processively along a microtubule by taking consecutive 8 nm steps by moving two motor heads in a coordinated manner. Recent studies showed that kinesin moves in a hand-over-hand manner, however, there is still a controversy on the structural changes that enable kinesin to exchange two heads. Rice et al (1999) have reported that the neck linker, a short stretch that connects motor head with the coiled-coil, undergoes ATP-dependent conformational changes (docked or undocked onto/from the head). Based on the results, they proposed a model that docking of the neck linker onto the microtubule-bound head moves partner head and facilitate stepping toward plus end of the microtubule. To test this idea, we observed structural changes of the neck linker during the movement using single molecule fluorescence resonance energy transfer (FRET) technique. Two reactive cysteines were introduced before and after the neck linker of one of the heads in kinesin heterodimer and were labelled with Cy3 and Cy5 dyes. When kinesin was bound to the microtubule in the presence of AMPPNP, two populations in the FRET efficiency that correspond docked and undocked states of the neck linker were observed. During the movement under low ATP concentration, FRET efficiency changed alternately between these two states. These results provide direct evidence that the neck linker of kinesin undergoes structural changes during the movement.

Structural studies of microtubule-based motors.

Masahide Kikkawa.
Cell Biology, Univ. of Texas, Southwestern, Dallas, TX, USA.

Kinesin is an ATP-driven microtubule-based motor fundamental to organelle transport. Although a number of kinesin crystal structures have been solved, the structural evidence for coupling between the bound nucleotide and theconformation of kinesin is elusive. In addition, the structural bases of themicrotubule-induced ATPase activity of kinesin is not clear because of theabsence of the microtubule in the structure. Here, we report cryo-electron microscopy structures of the monomeric kinesin KIF1A-microtubule complex in two nucleotide states at about 10 A-resolution, sufficient to reveal the secondary structure. This high-resolution maps visualized clear structural changes that suggests a mechanical pathway from the nucleotide to the neck linker via the motor core rotation. In addition, new nucleotide binding pocket conformations are observed that are different from X-ray crystallographic structures; it is closed in the AMPPNP state, but open in the ADP state. Based on these results, we propose a novel structural model of the thermal ratchet movement of kinesin, namely, "biased-capturing model".

Meredith Betterton, University of Colorado, Boulder
Poster Session II highlighted posters

One of the far-reaching implications of the work presented at this meeting on stepping and force generation in vitro is how the motors function in the cell, including their directional movement, regulation, and force production. These topics, which are being addressed in the second part of the meeting, represent the areas about which we know the least - how the motors step directionally, how they are regulated and how they produce force in the cell - and may require new methods to investigate. These areas represent cutting-edge motor research and present significant technical challenges. Work presented in the second poster session begins to address aspects of the problem of motor function and regulation in live cells, reporting new approaches to track motors and their cargo in the cell and to understand how motors work.

Observations by Kulic et al. show promising results in tracking specific vesicles carried by motors along microtubules. This poster, "Mechanisms of vesicle transport deduced from FIONA measurements in living cells," (#144) presents an intriguing observation of the effects of traffic jams in cells. The authors track fluorescently labeled peroxisomes, which are vesicle-like organelles filled with toxic substances, such as hydrogen peroxide. This poster presents observations on peroxisomes being transported by motors along microtubules inside thin protrusions extending from cells. The intriguing observation of Kulic et al. is the finding that the typical helical path taken by peroxisomes can be disrupted by crowding, an effect which, remarkably, can cause the peroxisome/motor complex to deform the microtubule track. The authors propose elastic deformation of microtubules as a possible mechanism of motor-motor interactions.

The poster by Peckham et al., "Functions of molecular motors in vivo," (#101) presents an interesting new result on the problem of how kinesin-transported cargo is directed to specific cellularlocations. They observe that when the kinesin motor Kif5C is fused to green fluorescent protein (GFP) and the chimera is expressed in live cells, The GFP-Kif5C fusion proteins move only along a subset of cellular microtubules. The authors then report that those microtubules which are used for transport bind a fluorescent antibody for detyrosinated tubulin, suggesting that addition and removal of tyrosines from tubulin may be used in cells to control which microtubules are used for transport. Peckham et al. then compared Kif5C in vitro and suggest that the kinesin binding occurs with higher affinity to detyrosinated microtubules. This is interesting because detyrosinated microtubules are more stable than tyrosinated microtubules, turn over more slowly, and have been reported to regulate intermediate filament interactions with microtubules in a kinesin-dependent fashion.

The poster by Cappello et al., "Kinesin/Microtubule (un)binding dynamics depends on the bulk kinesin concentration," (#177) presents interesting evidence for cooperative effects in the binding/unbinding of kinesin motors to/from microtubules. The authors used total internal reflection fluorescence (TIRF) microscopy to image kinesin molecules bound to microtubules. The study was performed with a non-hydrolyzable ATP analogue (AMPPNP), which means that bound kinesin motors should not move along the

microtubules. The main result is the observation of a spatially nonuniform - and highly concentration dependent - distribution of kinesin density along the microtubule axis. This uneven distribution has implications for motor function in live cells.

The poster by Catalano et al., "Mechanistic studies on a viral DNA packaging motor complex," (#94) gives new insight into the mechanism of terminases, the motors which package viral DNA into viral capsids. These motors sometimes work against a significant pressure which opposes DNA packaging. The Catalano et al. work uses kinetics characterization and biophysical methods to study the bacteriophage lambda terminase heteroligomer. (This terminase includes both a gpNu1 subunit which recognizes viral DNA and a gpA subunit with nuclease, helicase, and DNA translocase activity.) The results of interest include (a) suggested interactions between different catalytic sites in the enzyme, (b) a proposed kinetic model for terminase assembly, and (c) a proposed stoichiometry of assembly where four heterotrimers assemble into a ring-like complex that may encircle the DNA. Similar mechanisms may be operative for DNA packaging enzyme complexes, even in dissimilar viruses.

The poster by Neuman et al., "Chiral discrimination by Topoisomerase IV: crossing angle and processivity," (#185) addresses the interesting problem of topisomerase chiral preference. Like other topoisomerases, Topo IV removes supercoils from DNA, but Topo IV shows higher activity when removing positive supercoils (as opposed to negative supercoils). One proposed mechanism for this chiral sensing sensitivity of the enzyme to the crossing angle between DNA segments, which would differ (on average) between positive and negative supercoils. Neuman et al. use single-molecule measurements with braided DNA constructs to determine the enzymatic rate as a function of crossing angle. They report a surprisingly weak dependence of Topo IV unlinking on crossing angle. Instead, they suggest that the Topo IV processivity is different on positively and negatively supercoiled DNA. This work gives new insight into how a single enzyme can affect a large-scale topological property important in the cell (DNA supercoiling) while only acting locally.

Further insights into motor function in cells will come from sensitive particle tracking in three dimensions and detailed mechanistic studies of motors, as presented in this poster session, in addition to identification of additional motors in cells (a problem that may require RNAi together with genetics and antibody methods) and the development of new techniques to measure force inside cells.

Mechanistic Studies on a Viral DNA Packaging Motor Complex

Carlos Enrique Catalano, **Pharm.D.**, **Ph.D.**[1], Qin Yang, Ph.D.[2], Nasib Karl Maluf, Ph.D.[3], Helene Gaussier, Ph.D.[2].
[1]University of Washington School of Pharmacy, Seattle, WA, USA, [2]Pharmaceutical Sciences, University of Colorado School of Pharmacy, Denver, CO, USA, [3]Microbiology, University of Iowa, Iowa City, IA, USA.

Terminase enzymes are common to both eukaryotic and procaryotic double-stranded DNA viruses and serve as molecular motors that selectively "package" viral DNA into a pre-formed procapsid structure. Bacteriophage lambda terminase is a heteroligomer composed of gpA and gpNu1 subunits. The gpNu1 subunit is required for specific recognition of viral DNA, a process that is modulated by ATP. The gpA subunit possesses site-specific nuclease and helicase activities that "mature" the viral genome prior to packaging. The subunit further possesses a DNA translocase activity that is central to the packaging motor complex. Discrete ATPase sites in gpA modulate the DNA maturation reactions and fuel the DNA packaging reaction. Kinetic characterization of lambda terminase indicates significant interaction between the multiple catalytic sites of the enzyme and has led to a minimal kinetic model describing the assembly of a catalytically-competent packaging motor complex. The proposed model provides a unifying mechanism for allosteric interaction between the multiple catalytic sites of the holoenzyme, and explains much of the kinetic data in the literature. Biophysical studies demonstrate that purified lambda terminase forms a homogenous, heterotrimeric structure consisting of one gpA subunit in association with two gpNu1 proteins ($gpA_1 \bullet gpNu1_2$). Four heterotrimers further assemble into a ring-like complex with subunit stoichiometry $[(gpA_1 \bullet gpNu1_2)_4]$ and of sufficient size to encircle duplex DNA. The ensemble of data suggests that the ring tetramer represents the biologically relevant, catalytically-competent motor complex responsible for genome processing and packaging reactions. Given that similar packaging mechanisms have been proposed for viruses as dissimilar as lambda and the herpesviruses, the model may find general utility in our global understanding of the enzymology of virus assembly.

Functions of molecular motors in vivo

Michelle Peckham[1], Sarah Dunn[1], Ewan Morrison[2], Rob Cross[3].
[1]University of Leeds, Leeds, United Kingdom, [2]St James, CRUK, Leeds, United Kingdom, [3]MCRI Molecular Motors Group, Marie Curie Institute, Oxted, United Kingdom.

The mechanism of directed trafficking of kinesin dependent cargoes in vivo is unknown. We have found that de-tyrosinated microtubules act to direct kinesin trafficking to specific sites in the cell. GFP-Kif5C labelled puncta in live cells move along a subset of microtubules at rates of up to 1 micron per second towards the periphery and exchanged into cortically localised clusters at microtubule ends. This microtubule subset immunostained positively for detyrosinated tubulin. A rigor mutant GFP-Kif5c decorates centrally located microtubules in the centre of the cell and did not move or exchanging into cortical clusters. A YFP-Kif5c dimeric head construct did not bind and move along microtubules and had a dominant negative effect on microtubule organisation. Why does kinesin preferentially bind to de-tyrosinated microtubules in cells? In vitro, we found that Kif5c moved faster along microtubules assembled from unmodified tubulin (0.66 micron per second), than along microtubules composed of detyrosinated tubulin (0.58 micron per second). We propose that the preferential localisation of GFP-Kif5C along detyrosinated microtubules within living cells reflects a higher affinity of the motor for microtubules bearing this modification, leading to slower but more processive transport of Kif5C cargo to the ends of these microtubules. This suggests that recently defined signalling pathways shown to influence microtubule modification could play a role in targeting kinesin-1 dependent trafficking to specific intracellular sites.

Mechanisms of vesicle transport deduced from FIONA measurements in living cells.

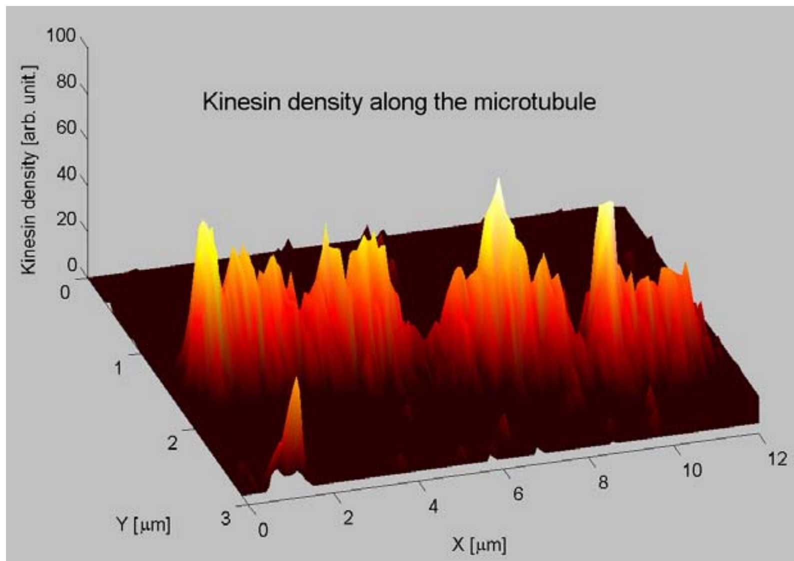
Igor Kulic[1], Comert Kural[2], **Philip Nelson, PhD**[1], Paul Selvin, PhD[2].
[1]Physics/Astronomy, Univ Pennsylvania, Philadelphia, PA, USA, [2]Physics, Univ Illinois, Urbana, IL, USA.

Fluorescence Imaging at One Nanometer Accuracy has recently been extended to observe the motion of single fluorescently labeled peroxisomes in living cells, where millisecond time resolution is critical. Two of us previously observed the peroxisomes walking along microtubules in thin processes extending from the cells. Here we report that (1) peroxisomes are sometimes observed to take well defined helical paths as they move down their track; (2) when prevented by cellular crowding from taking such tracks, their passage elastically deforms the track; (3) such deformation presents an opportunity for a novel cooperative-motor mechanism, and indeed multiple peroxisomes observed on the same microtubule are seen to have remarkable cross-correlations in their motion. Finally, we find that (4) the very rapid motions recently observed in this system have the time course expected from elastohydrodynamics of a torsionally stressed rod (the microtubule track). We also review other evidence in favor of our hypothesis that cargo walking along a microtubule track can torsionally strain the track, and outline consequences for intracellular transport.

Kinesin/Microtubule (un)binding dynamics depends on the bulk kinesin concentration.

Giovanni Cappello[1], Wouter Roos[1], Otger Campas[1], Fabien Montel[1], Patricia Bassereau[1], Günther Woehlke[2].
[1]Physico-Chimie-Curie, Insitut Curie, Paris Cedex 05, France, [2]Zellbiologie, Adolf-Butenandt-Institut (Ludwig-Maximilians-Universität München), D-80336 München, Germany.

We have observed the binding/unbinding dynamics between the kinesins and the microtubules in presence of AMP.PNP (non hydrolizable), in such a way that the molecular motors can only attach or detach (no directed motion). The kinesin distribution along the microtubules was observed by Total Internal Reflection Fluorescence (TIRF), as a function of the time. Our experiments have shown that the motors are not homogeneously distributed along the microtubules: this effect strongly depends on the bulk concentration of the kinesins. Those results are not compatible with a purely random (un)binding rate and suggest that their reciprocal interactions affect the attachment (k_{on}) and/or the detachment rate (k_{off}).



Chiral discrimination by Topoisomerase IV: Crossing Angle and Processivity

Keir C. Neuman, Gilles Charvin, Vincent Croquette, David Bensimon.
Physique Statistique, Ecole Normale Superieure, Paris, France.

Topoisomerase IV (Topo IV) resolves inappropriate topological states that result from DNA replication by removing positive supercoils and unlinking catenated daughter strands. Topo IV preferentially relaxes positive supercoils over negative supercoils. A chiral sensing mechanism, whereby the activity of Topo IV depends on the crossing angle between juxtaposed DNA segments, has been proposed to explain this asymmetric relaxation of supercoils. Single-molecule magnetic manipulation experiments permitted us to directly test this angle-dependent hypothesis by measuring unlinking rates of single left- and right-handed DNA crossings with well defined crossing angles. In conjunction with Monte Carlo simulations of the DNA crossings, these measurements yielded the preferred crossing angle for Topo IV. Surprisingly, the preferred crossing angle between juxtaposed DNA segments is significantly closer to symmetry than previous estimates and can not account for the difference in relaxation rates between positive and negative supercoiled DNA. Real-time measurements of supercoil relaxation by Topo IV, however, reveal a marked difference of processivity between positive and negative supercoiled substrates. Based on these findings, we propose a model for supercoil relaxation by Topo IV in which a slight asymmetry in crossing angle preference in conjunction with a large asymmetry in processivity produces the overall difference between positive and negative supercoil relaxation rates.

Minimum requirements for processivity in kinesin-1

Sarah Adio, Guenther Woehlke.
Department of Cell Biology, University of Munich (LMU), Munich, Germany.

Members of the kinesin superfamily are involved in a variety of different cellular transport processes along the microtubule cytoskeleton. These motors are processive *e.g.* they take multiple consecutive steps on microtubule filaments. Processive motors are thus capable of supporting continuous and directed transport of cargo. A single processive kinesin is sufficient to transport cargo over µm distances to its final destination. In constrast, effective transport of cargo by non-processive kinesins relies on large ensembles of motor proteins that work in concert. However, comparison of their respective domain organisation reveals no fundamental differences. In both types of motors two identical polypeptide chains are joined via coiled-coil interactions of the C-terminal tail domain to form the so called stalk. The homodimeric heads are connected to this stalk via a flexible neck linker domain.
To find out the structural determinants for processive movement and to define a minimal processive motor, we compared a processive kinesin-1 motor with a non-processive member of the kinesin-3 family. To this end, we generated chimeric kinesins consisting of the motor domain of the processive kinesin -1 that is connected to the neck linker and the stalk of the non-processive motor and vice versa. Additionally, we connected motor domain plus neck linker of the processive motor to the coiled-coil domain of the non-processive enzyme.
Single-molecule, steady state and pre-steady state methods were applied to assess whether the chimera show processive or non-processive motion. Interestingly, the ability to perform processive motion seems to be an intrinsic feature of the motor core as neither connection by the coiled-coil of the non-processive kinesin nor by an artificial stable coil domain can prevent kinesin-1 motor heads from walking processively along the microtubule.

Electron-microscopy and fluorescence polarization studies on motors of the kinesin superfamily.

Hernando J. Sosa, Dongyan Tan, Ana Belen Asenjo, Yonatan Weinberg, Vito Mennella, David Sharp.
Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY, USA.

We have used electron microscopy and fluorescence polarization microscopy (ensemble & single molecules) to obtain structural and functional information on the interaction of kinesin proteins with the microtubules. Fluorescence polarization microscopy of conventional kinesin (kinesin-1) with probes attached to the core motor domain or the neck-linker region supports a model in which both motor domains of the dimer are attached in similar configurations to the microtubule for most of the ATPase cycle. The neck-linker region of monomeric and dimer constructs on the other hand is very mobile in the absence of nucleotides and becomes less mobile and align along the microtubule axis in states mimicking ATP binding (AMPPNP) or hydrolysis (ADP+AlF4-). The largest population of ordered neck-linkers is found with monomeric constructs in the presence of ADP+AlF4-. We also found that the configuration of the docked neck-linkers is different in monomers and dimers. These results support the role of ATP binding on neck-linker docking and suggest a mechanism of communication between the two motor domains through their neck-linkers connection. In a parallel study we have used electron microscopy to investigate possible differences in the way that proteins of kinesin-13 family interact with microtubules. Kinesin-13s have the distinct property among the kinesins of inducing microtubule depolymerization rather than moving along them. We have found that kinesin-13s are also unique in the way that they interact with the microtubule. Kinesin-13s can form oligomeric rings and spirals around the microtubule. Possible implications of this finding will be discussed.

The mechanics of myosin V

Michael A. Riegelman[1,2], James R. Sellers, Ph.D.[2], Haim H. Bau, Ph.D.[1].
[1]Mechanical Engineering & Applied Mechanics, University of Pennsylvania, Philadelphia, PA, USA,
[2]Molecular Physiology, National Heart, Lung, and Blood Institute, NIH, Bethesda, MD, USA.

In recent years myosin V has become the most highly characterized actin-dependent motor protein. Structurally, myosin V has two motor domains connected to unusually long neck domains which are tethered via a coiled-coil. Its biochemical cycle is dominated by states with high actin affinity. The combination of these kinetic and structural features allows myosin V to move processively, however the extent of the processive motion is determined by intramolecular strain between the motors coordinating the chemomechanical cycle. To gain insight into this "gaiting mechanism" we constructed a purely mechanical model for myosin V. Based on structural data, electron microscopy images, and various single molecule studies, myosin V's neck was modeled as an elastic cantilever connected to the motor via a pliant joint (acting as a torsional spring) and tethered through a frictionless hinge. The model was used to characterize how intramolecular strain between the motors is transmitted to the nucleotide binding pocket and analyze how these loads coordinate the chemomechanical cycle. Our analysis provides insights into: 1) results from optical-tweezer based studies on myosin V's load dependent kinetics; 2) single-molecule based observations on myosin V's step size; and; 3) electron microscopy images of the structural conformation of myosin V's in its strained conformation.

Interaction of dynein and microtubules

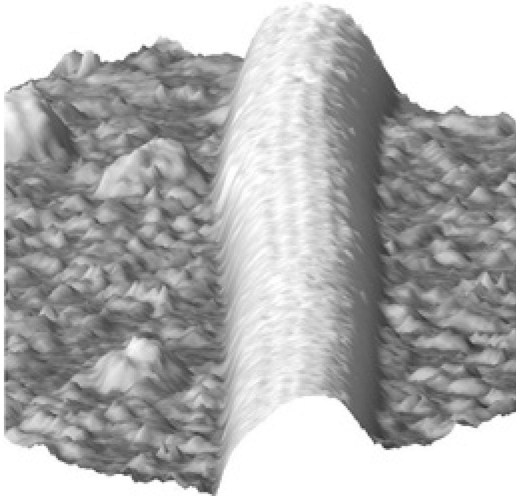
Yoko Y. Toyoshima, PhD.
Lifesciences, The University of Tokyo, Tokyo, Japan.

Dynein stalk head binds to a specific region on tubulin dimer of microtubules and this binding region is overlapped with that for kinesin (Mizuno et al. 2004). These regions are located along one side of the protofilament surface different from the side of MAPs binding. However, it is not clear yet whether dynein uses a single protofilament or two neighboring protofilaments when walking on microtubules. To answer this question, we made zinc-induced sheets and macrotubes and examined the dynein motility. A zinc-sheet has an arrangement of adjacent protofilaments with anti-parallel and opposed orientations. A zinc-macrotube has a cylindrical structure to which a zinc-sheet rolls up. Consequently, a zinc-sheet has two edges along protofilaments, whereas a zinc-macrotube has no edge. In the dynein motility assay, zinc-sheets moved well at almost the same speed as microtubule movement and showed the winding paths different from microtubule movement. On the other hand, zinc-macrotubes did not bind dynein at all. From the structural aspect, the dynein binding region on tubulin seems to be buried in the interface between two juxtaposed protofilaments of zinc-macrotube. The dynein binding region should be exposed along a single protofilament at one edge of zinc-sheet, and thus, dynein can bind one edge of zinc-sheet. Our motility results suggest that a single protofilament is enough to support dynein movement and imply that dynein moves along single protofilament as kinesin does.

Microtubules and Microtubule-binding Proteins studied with Atomic Force Microscopy

Iwan A.T. Schaap, PhD[1,2], Carolina Carrasco[3], Bernd Hoffmann, PhD[4], Pedro J. de Pablo, PhD[3], Frederick C. MacKintosh, PhD[2], Christoph F. Schmidt, PhD[2,5].
[1]Physical biochemistry, NIMR, London, United Kingdom, [2]Department of Physics and Astronomy, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands, [3]Departamento de Física de la Materia Condensada C-III, Universidad Autónoma de Madrid, Madrid, Spain, [4]Institute of Thin Films and Interfaces, Research Center Jülich, Jülich, Germany, [5]III. Physikalisches Institut, Faculty of Physics, Georg-August-Universität, Göttingen, Germany.

We have used atomic force microscopy (AFM), to image single microtubules (MTs) in buffer with nanometer resolution. Key parameter is the force applied to the sample by the AFM tip. We found forces up to 100 pN suitable to scan MTs without deforming them, while at forces around 0.3 nN we found (sometimes reversible) disruption of protein bonds. In addition we used the AFM tip to accurately probe the mechanical response of MTs upon indentation. To measure the effects of microtubule binding proteins on the MT stability, we decorated MTs with tau or kinesin and probed the mechanical response of the decorated MTs.
Because AFM couples single-protein resolution to the ability to work in physiological conditions we set out to use AFM to follow the dynamics of MT binding proteins. Ultimately this would allow us to investigate properties of these proteins that are not accessible by any of the other currently available single-molecule techniques. We hope to present the first results during the biophysical discussions 2006.



Tracking of Single Kinesin-1 Motor Molecules in Live Cells

Dawen Cai[1], Kristen J. Verhey[2], Edgar Meyhofer[3].
[1]Biophysics Research Division, University of Michgan, Ann Arbor, MI, USA, [2]Cell and Developmental Biology, University of Michgan, Ann Arbor, MI, USA, [3]Mechanical Engineering and Biomedical Enginerring, University of Michgan, Ann Arbor, MI, USA.

The organization and functional properties of eukaryotic cells depend on intracellular transport processes driven by molecular motors. Microtubule-based kinesin motors power a wide variety of these transport processes, including intracellular trafficking and mitosis, and play important roles in neurodegenerative diseases. Although tremendous progress has been made in understanding the structural and motile properties of single kinesin-1 motors in vitro, how the motor functions in vivo is unknown. This is particularly important for understanding cellular transport as the motile properties of organelles and vesicles in vivo cannot be explained by the motile properties of kinesin-1 in vitro . Here, we investigate for the first time the motility of single kinesin molecules in live cells. Kinesin-1 fused to three tandem copies of the monomeric fluorescent protein Citrine (3XmCit) was imaged in live COS cells by low background total internal reflection fluorescence microscopy (TIRFM). The quantized bleaching behaviour of individual fluorescent spots confirmed the tracking of single, dimeric kinesin-1 motors. In vivo these kinesins moved with an average velocity of 0.8 μm/s, and the processive run length of individual motile events was 1.2 μm. Virtually identical in vitro motile properties were obtained for 3xmCit-labelled kinesin-1 extracted from cells. These results demonstrate that the motile ability of individual motors is not up-regulated in vivo nor hindered by macromolecular crowding. In addition, these results provide the first direct observations of a single protein expressed and localized in the cytoplasm of mammalian cells.

THE EFFECT OF MONASTROL ON EG5/DMKHC CHIMERAS

Stefan Lakämper[1,2], Mikahil Korneev[2], Christoph F. Schmidt[1,2].
[1]Drittes Physikalisches Institut, Georg-August-Univerität Göttingen, Göttingen, Germany, [2]Physics of Complex Systems, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands.

Native Eg5 is a homo-tetrameric motor, which is essential for the formation of the mitotic spindle in Xenopus laevis egg-extracts. Due to the class-specific formation of anti-parallel coiled-coils of the neck and stalk domains, the extended tetramer exposes two motor domains at each end, reminiscent of conventional kinesin. While it was difficult to test for the processivity and force generation of specifically only one end, native tetramers were observed to produce low forces, possibly indicating regulatory effects mediated by the proximal tail/BimC-domain. Only very recently the problems to obtain a stable dimeric Eg5-motor by mere truncation have been overcome and allowed to show that Eg5-dimers move processively and produce high forces. Here, we present an alternative approach using dimeric Eg5-head/DmKHC-tail chimeras to investigate details of Eg5-motility. The speed of and force produced by single chimeras is similar to native, truncated dimers, but we observe a much higher run length. Interestingly, and in contrast to current models, we observe a sharp exponential decrease in processivity, but not speed by the small Eg5-specific inhibitor, Monastrol. We expect that further chimeric constructs will allow us to gain further insight into the molecular mechanisms important for Eg5-function and regulation within the context of the spindle apparatus.

Motility and Processivity of Kinesin-2 Motors

William O. Hancock, PhD, Yangrong Zhang, PhD, Gayatri Muthukrishnan, MS.
Bioengineering, Pennsylvania State University, University Park, PA, USA.

Kinesin-2 motors, which are involved in intraflagellar transport as well as transport along cytoplasmic microtubules, have two different heads, while Kinesin-1 motors (conventional kinesin) have two identical heads. To understand how their motor properties relate to their different intracellular roles, we compared the processive run lengths and microtubule affinity of KIF3A/B (the mouse Kinesin-2 ortholog) and DmKHC (Drosophila conventional kinesin). Using objective-based total internal reflection fluorescence microscopy of GFP-labeled motors, we found that KIF3A/B was four times less processive than DmKHC. The KD for microtubules was compared for the two motors using fluorescence correlation spectroscopy (FCS). As expected, free motors and microtubule-bound motors were found to have different characteristic diffusion times in solution. By fitting the correlation curves to two time constants, the fraction of free and microtubule-bound motors were determined over a range of microtubule concentrations and used to calculate the KD for microtubules. Using these affinities together with the off-rates measured from the run-length data, we found that the on-rate for microtubule binding was 3-times slower for KIF3A/B than for DmKHC. This slow on-rate may imply that KIF3A/B has a preference for axonemal microtubules over the cytoplasmic microtubules used in these experiments. Our next experiment was to test whether the processivity of KIF3A/B is dependent on having two different head domains. Homodimeric KIF3A/A-GFP and KIF3B/B-GFP were constructed and tested in the processivity assay. Their run lengths were similar to wild-type KIF3A/B-GFP. Hence, two different heads are not necessary for KIF3 processivity.

Experimental Realization of Molecular Motor Models

Benjamin J. Lopez, B.S Physics completed, PhD in progress, Erin Craig, B.S Physics completed, PhD in progress, Heiner Linke, PhD.
Physics, University of Oregon, Eugene, OR, USA.

Various types of linear molecular motors, for example Kinesin and Myosin-V, share many physical features: they exist in a thermal environment where Brownian motion plays an important role, the heads are coupled, they are processive, and the heads interact with a track. We construct an experiment to implement a generalized model of the motor step, based on important physical features observed in biological motors. A dual scanning line optical trap system is used to create free-energy landscapes that replicate key mechanical stages of the motor step. This optical free-energy landscape can be modified to simulate varying degrees of power stroke and diffusive stepping. Feedback control allows position-sensitive binding/unbinding probabilities to be applied. The trap line separation can be modified so as to vary the coupling strength between the motor heads. These parameters can be tuned to a more fine degree than in current experiments using actual biological motor molecules, so that their effect on motor performance can be tested.
In a scanning line optical trap the beam is quickly scanned, back and forth, in one-dimension over a length tens of microns long. The trapped particle feels a time-averaged potential in that dimension, and strong trapping forces in the other two. The two-headed motor construct can be created through the coupling of two, micrometer scale, polystyrene beads by DNA. This size scale is large enough to easily track the motion of the beads using video microscopy, while small enough to allow Brownian motion to play a significant role. We are investigating how these parameters affect the stepping motion of our artificial motor in order to compare these features with biological molecular motors. This project is the experimental analogue of the numerical modeling that we are also doing. (See poster presented by Erin Craig.)

Kinesin motion induced by nucleotide analogs reveals a mechanism for head coordination

Nicholas R. Guydosh[1], Steven M. Block[2].
[1]Biophysics, Stanford University, Stanford, CA, USA, [2]Biological Sciences and Applied Physics, Stanford University, Stanford, CA, USA.

The kinesin motor generates processive motion over long distances. This movement can only occur if the kinetic cycles of the two heads are maintained out of phase (‘gated’) to ensure that at least one head remains tightly bound to the microtubule at all times. Two broad classes of models have been advanced to explain this gating: these both invoke the effect of intermolecular strain generated during the cycle at the point where both heads are bound to the microtubule. In one class of models, strain inhibits the front head until the back head catches up (Hancock & Howard, 1999; Schief, W.R. *et al.*, 2004). In the other class, strain releases the back head before the front head proceeds prematurely (Rosenfeld, S.S. *et al.*, 2003; Klumpp, L.M. *et al.*, 2004). We tested these alternatives at the single-molecule level by probing kinesin kinetics using analogs of ATP and inorganic phosphate. Steps taken by native squid kinesin molecules bound to beads under optically force-clamped conditions were scored in the presence of ATP and beryllium fluoride (BeF_x). Binding of BeF_x interrupts normal stepping by inducing long pauses. Our data are consistent with BeF_x binding to the rear head before ATP can bind to the front head to trigger a normal forward step. During long pause events, we were able to resolve numerous short-lived backsteps of 8 nm. We found that normal forward stepping could only resume after a final, obligatory backstep, during which BeF_x was released from the front head. We conclude that the affinity of the front head for BeF_x is reduced by strain, supporting the model where strain leads to processivity by inhibiting the front head from productively binding ATP and triggering a mechanical step.

Polymer-based Brownian motors with application to biomolecular sysytems

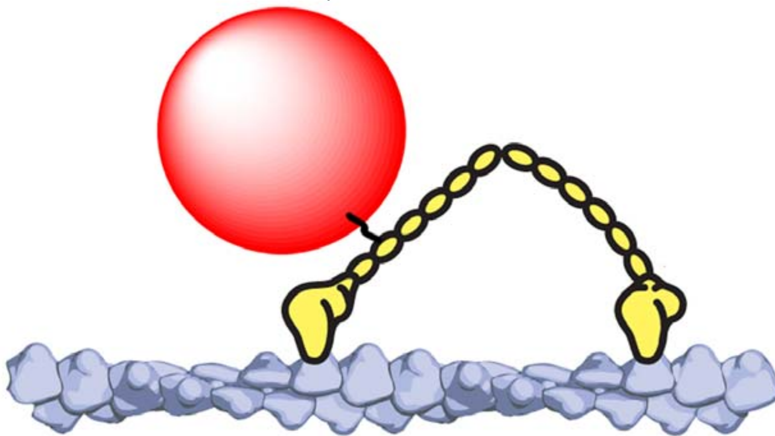
Martin J. Zuckermann, D. Phil..
Physics, Simon Fraser University, Burnaby, BC, Canada.

My colleagues and I work with simple molecular models with a view to understanding the behavior of real molecular motors. We used numerical simulation to study a single polymer chain in a flashing ratchet potential to determine how the mechanism of this Brownian motor system is affected by the presence of internal degrees of freedom. The polymer is modeled by a freely jointed chain with N monomers. Each monomer is acted upon by an asymmetric 1D piecewise linear potential with spatial period, L, of the same order as radius of gyration of the polymer. This potential is characterized by a localization time, t_{on}, and by a free diffusion time, t_{off}. We calculate the average motor velocity as a function of L, t_{off}, and N to determine optimal parameter ranges, and we evaluate motor performance in terms of finite dispersion, Peclet number, rectification efficiency, stall-force, and transportation of a load against a viscous drag. We find that the polymer motor performs qualitatively better than a single particle in a flashing ratchet: with increasing N, the polymer loses velocity more slowly than expected in the absence of internal degrees of freedom, and the motor stall force increases linearly with N. We investigate the cooperative aspects of motor operation by studying the relevant Rouse modes. The experimental feasibility is analyzed, the parameters of the model are scaled to those of lambda-DNA and the relevant experimental results are presented. In the context of our experimental realization, we present modeling results for a 2D flashing ratchet constructed using an electrode array, and find good agreement with the results of 1D simulations. Our ongoing simulation project extends our model to a multi-polymer sytems with a view to understanding molecular motors such as myosin in muscle fibers.

Tracking Single Gold Nanoparticle-Myosin V Conjugates Using Darkfield Imaging.

Alexander Robert Dunn, **Ph.D.**, L. Stirling Churchman, B.S., Zev Bryant, Ph.D., James A. Spudich, Ph.D..
Biochemistry, Stanford University, Stanford, CA, USA.

We describe experiments wherein we track the motion of a small (30-60 nm) gold particle attached to one of the two lever arms of dimeric myosin V. Gold nanoparticles scatter visible light extremely efficiently, making it possible to track their motion under darkfield imaging with sub-millisecond time resolution. In addition, the asymmetric attachment of the gold particle allows us to directly observe elements of the catalytic cycle that must be inferred from optical trapping experiments, which follow the myosin V center of mass. We present data tracking gold nanoparticle-myosin V conjugates with high temporal resolution, and discuss the mechanistic implications of our results.



Myosin VI Walks Wiggly on Actin Filaments

Yujie Sun[1], Harry W. Schroeder[1], John F. Beausang[1], Kazuaki Homma[2], Mitsuo Ikebe[2], Yale E. Goldman[1].
[1]Physiology, Pennsylvania Muscle Institute, Philadelphia, PA, USA, [2]University of Massachusetts, North Worcester, MA, USA.

Myosin VI is a motor protein that moves processively on actin filaments. To determine its walking mechanism, we measured orientation changes of bound bifunctional rhodamine-labeled CaM during processive motility using single molecule fluorescence microscopy. The azimuthal angle of the lever arm relative to actin often showed abrupt changes indicating twisting of myosin VI around the actin axis and large azimuthal flexibility. The rate of these tilts increased with [ATP]. A gliding filament assay showed that myosin VI caused actin to twirl with a right-handed pitch of about 1.0 μm. These azimuthal changes and their variability suggest a mechanism in which myosin VI can search for an actin binding site almost ±90° around the filament axis. Supported by NIH grant AR26846.

Processive Run Lengths Of Myosin V Are Determined By Its Affinity For Actin In The Weak-Binding State

Alex R. **Hodges**, Elena B. Krementsova, Kathleen M. Trybus.
Molecular Physiology and Biophysics, University of Vermont, Burlington, VT, USA.

The processive motor myosin V has a high affinity for actin in the presence of ATP compared to non-processive myosins. Is this feature required for myosin V to walk processively for long distances along an actin filament? We have previously shown that alterations in loop 2, which is involved in the electrostatic interaction between actin and myosin, can affect both the actin affinity and processive run length (Krementsova et al. (2006), JBC **281**, 6079). To study this question more systematically, we engineered a series of mutants with altered charge in loop 2. These mutations affect actin affinity in myosin V without affecting other steps in the ATPase cycle, as shown previously (Yengo and Sweeney (2004) Biochemistry **43**, 2605) and confirmed in our laboratory. Single molecule motility measurements using TIRF microscopy show that a mutant with a lower actin affinity has the same velocity as the wild type, but exhibits a dramatically reduced processivity, with a five-fold lower characteristic run length. We hypothesize that a high actin affinity allows the detached head of a stepping myosin V to find its next binding site faster, and stabilize it until it transitions to a strong binding state, thus decreasing the probability of termination during stepping. We are currently testing mutants with increased actin affinity to determine if they have increased processive run lengths, or if the actin affinity of the wild type is optimal.

Toward a Coarse-Grained Model of the Myosin V Neck Using the Normal Mode Analysis

david parker, **masters science**.
Mechanical engineering, stanford university, Stanford, CA, USA.

Myosin V is a two-headed motor protein that moves along single actin filaments transporting cellular cargoes within the cytoplasm. The neck region of myosin V is a long, light-chain-binding helix extending from each head. The neck is critical to myosin V movement, acting as a lever arm and is thought to act as an elastic element and coordinating the chemical states of its heads.
We constructed two novel atomic-resolution models of a chicken brain myosin Va monomer, comprising the head and neck regions with six bound light chains, in order to study its mechanical properties. Models were constructed from known partial X-ray crystal structures. The first model used all light chains in a compact conformation, and the second used two of the six light chains in extended conformations based on the IQ sequences. An elastic network based normal mode analysis was performed on these models to identify global motions of the neck. Examination of low frequency modes showed that for the compact light chain model, pairs of light chains spaced 12 residues apart moved collectively, regions of flexibility were identified where the light chains were spaced 14 residues apart. The model with extended light chains shows collected motion for groups of three light chains about the region of the extended light chain. These results demonstrate that as few as three mechanical elements can be used as a coarse-grained model for the neck.

Regulation of Microtubule-binding Affinity in Dynein

Ian R. Gibbons, Joan E. Garbarino, Andrew Hahn.
Mol/Cell Biology, University of California Berkeley, Berkeley, CA, USA.

The microtubule binding domain (MTBD) of dynein is separated from the AAA core of the motor by an ~15 nm stalk that is predicted to consist of an anti-parallel coiled coil. The mechanism by which this coiled-coil mediates communication between the MTBD and ATP-binding core is not known. To identify the optimal alignment between the hydrophobic heptad repeats in the two strands of the stalk coiled-coil, we fused the MTBD of dynein, together with 12-36 residues (~25%) of its stalk, onto a stable coiled-coil base, provided by Thermus thermophilus seryl tRNA-synthetase (SRS). By assaying these chimeric constructs for microtubule binding in vitro, we have identified an optimal register with 22 residues and 19 residues in the upstream and downstream strands of the coiled coil, respectively, (SRS-22:19) that yields a protein possessing high-affinity (2.2 μM) for microtubules. Two approximately full-length stalk constructs with the same register of coiled coil strands (SRS-85:82 and SRS-99:96) show a similarly high affinity, suggesting that this register corresponds to the native tight-binding conformation of the MTBD. Other register alignments generated by adding or deleting one to seven residues from the upstream strand of the tight-binding constructs have ~10-fold lower affinity. Assay of the thermostability of these different weak-binding constructs shows that one particular register, corresponding to SRS-26:19 and SRS-89:82, is substantially more stable than any of the others tested, suggesting that it may correspond to the native weak-binding conformation of the MTBD. These data support the hypothesis that dynein utilizes small amounts of sliding displacement between the two strands of its coiled-coil stalk as a means of communication between the AAA core of the motor and the MTBD, with a displacement of a hemi-heptad in one strand corresponding to the MTBD switching between its tight- and weak-binding conformations.

Single alpha helices (SAH domains) in myosin motor function

Peter J. Knight[1], Kavitha Thirumurugan[1], Thomas Baboolal[1], Arnout P. Kalverda[1], Yuhui Xu[2], Fei Wang[2], Walter F. Stafford III[3], James R. Sellers[2], Michelle Peckham[4].
[1]Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, United Kingdom, [2]NHLBI, National Institutes of Health, Bethesda, MD, USA, [3]Analytical Ultracentrifugation Research laboratory, Boston Biomedical Research Institute, Boston, MA, USA, [4]Institute of Molecular and Cellular Biology, University of Leeds, Leeds, United Kingdom.

Myosin 10 contains a region of predicted coiled coil 120 residues long. However, the highly charged nature and pattern of charges in the proximal 36 residues appear incompatible with coiled-coil formation. We have shown by circular dichroism, NMR, and analytical ultracentrifugation that a synthesized peptide containing this region forms a stable single alpha-helix (SAH) domain in solution and does not dimerize to form a coiled coil even at millimolar concentrations. Additionally, electron microscopy of a recombinant myosin 10 containing the motor, the three calmodulin binding domains, and the full length predicted coiled coil showed that it was mostly monomeric at physiological protein concentration. In dimers the molecules were joined only at their extreme distal ends, and no coiled-coil tail was visible. Furthermore, the neck lengths of both monomers and dimers were much longer than expected from the number of calmodulin binding domains. In contrast, micrographs of myosin 5 heavy meromyosin obtained under the same conditions clearly showed a coiled-coil tail, and the necks were the predicted length. Thus the predicted coiled coil of myosin 10 forms a novel elongated structure in which the proximal region is a SAH domain and the distal region is a SAH domain, or has an unknown extended structure, that dimerizes only at its end. Sequence comparisons show that similar structures may exist in the predicted coiled-coil domains of myosins 6 and 7a and MyoM and could function to increase the size of the working stroke. We are investigating whether these sequences do indeed form SAH domains and how the SAH domain contributes to myosin function.

Step size adaptations in myosins V, VI and X.

Ronald S. Rock, Ph.D..
Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, USA.

Myosin V is a molecular motor with six IQ repeats in the neck region. These long lever arm domains allow processive stepping without spiraling, as the motor can span the actin pseudorepeat. Likewise, myosin VI takes processive, 36 nm steps, despite having only two bound light-chains per lever arm. Instead, myosin VI relies on a flexible linker that allows the two heads to separate to span 36 nm distances. These results suggest that myosins have multiple adaptations that allow 36 nm steps, and that spiraling is strongly disfavored. Here we show that forced dimers of myosin X, a myosin with three IQ domains, can follow a spiral path along an actin filament. Sliding actin filaments supercoil, forming micron-long plectonemes in some cases. The observed supercoils are consistent with a left-handed spiral stepping pattern, as would be expected if myosin X takes steps in the range of 18-36 nm.

Understanding Stall, Superstall, Back Steps and Dwell Times

Michael E. Fisher
University of Maryland

The Carter-Cross experiments on kinesin[1] observed runs of back steps and, hence, negative, i.e., backwards velocities. About 40% beyond the stall force, $F_S \simeq -8$ pN, the velocities exhibit shallow *minima.* Within the basic N-state kinetic model,[2] such minima imply[3] *geometric constraints* on the locations of the intermediate mechanochemical states of the stepping cycle relative to their transition states.[2] The velocity *maxima* found under *assisting loads* [1, 2, 4] entail similar constraints.[3]

The fraction of back steps, $\pi_- = 1 - \pi_+$, must approach 50% at stall: and for loads F near F_S both observation[1] and theory[5] agree that

$$\pi_+ / \pi_- \approx \exp[(F - F_S)\, d^* / kT].$$

But what value and significance has the 'effective step length' d^*? A first-passage analysis [6] should answer. The natural approach predicts $d^* = d$, the step size, namely 8.2 nm for kinesin; but experiment[1] yields $d^* \simeq 4$ nm ! The discrepancy arises because of "hidden substeps," i.e., spatially unresolved mechanochemical transitions.[5] Accounting for these[5] shows that d^* falls below d and carries transition-rate information.

The same analysis[5] describes correctly *pairwise step probabilities,* such as the fraction of forward/back steps followed by a forward step, π_{++}, and π_{-+}, etc., and the *conditional dwell times* τ_+ and τ_-, prior to forward/back steps, τ_{++} between successive forward steps, etc.

References: **(1)** Carter NJ, Cross RB (2005) Nature **435**, 308-312; **(2)** Fisher ME, Kim YC (2005) PNAS **102**, 16209-214; **(3)** Tsygankov D, Fisher ME (2006) unpublished; **(4)** Block SM *et al.* (2003) PNAS **100,** 2351-2356; **(5)** Lindén M, Tsygankov D, Fisher ME (2006) unpublished; **(6)** Kolomeisky AB, Stukalin EB, Popov AA (2005) Phys. Rev. E **71**, 031902.

Mechanical model for coordinated, processive transport of dimeric motors

Erin M. Craig[1], Ben J. Lopez[1], Martin J. Zuckermann[2], Heiner Linke[1].
[1]Physics Dept. and Materials Science Institute, University of Oregon, Eugene, OR, USA, [2]Department of Physics, Simon Fraser University, Burnaby, BC, Canada.

Single-molecule experiments have been used to observe the motion of the motor domains (heads) and the cargo of processive, dimeric molecular motors such as kinesin and myosin-V, within a single step of their hand-over-hand transport. We use information from existing experimental data about a motor's average trajectory, stepping behavior and fluctuations to theoretically model the mechanisms of force generation underlying the observed motion. We use a minimal, mechanistic model in which most parameters are constrained by experimental observations. By varying parameters that correspond to open questions about the transport mechanism, we predict qualitative performance features that can be tested experimentally.
In particular, we numerically simulate two coupled particles that are each exposed to a one-dimensional, time-dependent free energy landscape that switches stochastically among several discrete states. By tuning features of the free energy landscape, we explore the roles of diffusion, deterministic conformational change (power stroke), and cooperative motion of the two binding domains. We use a conformational feedback scheme to model the role of internal strain in the coordinated motion of motor domains. Model predictions are obtained through Langevin dynamics simulations as well as analytical solutions to the system's Fokker-Planck equations. We will use this model as the basis for experimental realizations of artificial molecular motors in which mechanically coupled beads are exposed to a time-dependent potential profile created with a scanning optical trap. This experimental setup will provide a powerful tool for studying the physics of motors that operate in an environment dominated by thermal fluctuations. (For more information about this experiment, see the poster presented by Ben Lopez.)

DIFFUSION-LIMITED PROCESSES IN MYOSIN FUNCTION

P. Bryant Chase, **Ph.D.**[1], Nicolas M. Brunet, B.S.[2], Goran Mihajlović, B.S.[3].
[1]Biological Science, Molecular Biophysics, Chem & Biomed Engr, Florida State University, Tallahassee, FL, USA, [2]Biological Science, Molecular Biophysics, Florida State University, Tallahassee, FL, USA, [3]Physics, Center for Materials Research & Tech (MARTECH), Florida State University, Tallahassee, FL, USA.

Increasing solvent viscosity (η) by adding low MW sugars (mono- and disaccharides) reversibly inhibits: (i) sliding speed (*s*) of unregulated F-actin in motility assays (1); and, in permeabilized fibers from rabbit psoas at saturating Ca^{2+}, (ii) maximum velocity of sarcomere shortening and (iii) kinetics of isometric tension redevelopment (k_{TR}) (2). These kinetic parameters vary inversely with η (1, 2) as expected for a diffusion-limited process. Increasing η did *not* inhibit maximum isometric force in fibers (2) or K-EDTA ATPase activity of heavy meromyosin (1) with the same, inverse linear relationship; inhibition of these two parameters occurred only at high [solute] (high η). Inibition of k_{TR}, which is determined under near-isometric conditions, indicates that η does not necessarily affect filament sliding *per se*, consistent with hydrodynamic calculations (3). Comparing mono- versus disaccharides indicates that the relevant inhibitory parameter is η and not osmolarity. Lack of effect of η on ATPase activity suggests that substrate binding may not be the limiting factor. To further investigate diffusion-limited processes in filament sliding, we varied temperature 20-60°C in motility assays (4). Arhennius analysis indicated two phases (two slopes) for regulated thin filament *s* (pCa 5), but only a single phase for unregulated F-actin. Correcting for temperature-dependent changes in η did not alter the number of phases. The shallower slope for regulated thin filaments was 27 kJ/mol, consistent with a diffusion-limited process.

1. Chase, P. B., Chen, Y., Kulin, K. & Daniel, T. L. (2000) *Am J Physiol Cell Physiol* 278:C1088-C1098.
2. Chase, P. B., Denkinger, T. M. & Kushmerick, M. J. (1998) *Biophys J* 74:1428-1438.
3. Huxley, A. (1980) Reflections on Muscle (Princeton University Press, Princeton, New Jersey).
4. Mihajlovic, G., Brunet, N. M., Trbovic, J., Xiong, P., von Molnár, S. & Chase, P. B. (2004) *Appl Phys Lett* 85:1060-1062.

The power stroke of myosin VI

Zev Bryant, David Altman, James A. Spudich.
Biochemistry, Stanford University, Stanford, CA, USA.

Myosin VI supports movement toward the (-) end of actin filaments[1], despite sharing extensive sequence and structural[2] homology with (+) end directed myosins. A class-specific insertion was proposed to provide the structural basis of directionality reversal[1]. Indeed, the unique insert mediates a ~120° redirection of the lever arm in the presumed post-stroke conformation of myosin VI crystallized by Houdusse and coworkers[2]. However, this redirection alone is insufficient to account for the large (-) end directed stroke of a myosin VI S1 construct[3]. Houdusse and coworkers therefore proposed models in which the underlying motion of the myosin VI converter domain differs substantially from the power stroke of (+) end directed myosins[2]. In *model I*, the converter domain moves toward the (+) end while the end of lever arm swings toward the (-) end. In *model II*, the converter transitions from an uncoupled state biased toward the (+) end into a docked state positioned toward the (-) end. In order to experimentally map out the motion of the converter domain and lever arm, we have generated a series of truncated myosin VI constructs and characterized the size and direction of the power stroke for each construct using dual-labeled gliding filament assays and optical trapping. Motors truncated near the end of the converter domain generate (+) end directed motion, whereas longer constructs move toward the (-) end. Our results support *model I* but are inconsistent with *model II*. We suggest that the lever arm rotates ~180° between pre- and post-stroke conformations.

1. Wells, A. L. et al. (1999) Nature 401, 505-8
2. Menetrey, J. et al. (2005) Nature 435, 779-85.
3. Rock, R. S. et al. (2005) Mol Cell 17, 603-9.

Function and regulation of Cin8p, the mitotic *S. cerevisiae* kinesin-5 homologue

Adina Gerson, M.Sc.[1], Rachel Avunie, M.Sc.[2], Natalia Movshovich, B.Sc.[1], Irena Gertsberg, Ph.D.[2], Vladimir Fridman, B.Sc.[2], Scot C. Kuo, Ph.D.[3], Myles Andrew Hoyt, Ph.D.[4], Benjamin Katz, Ph.D.[5], **Larisa Gheber, Ph.D**.[1].
[1]Clinical Biochemistry and Chemistry, Ben-Gurion University of the Negev, Beer-Sheva, Israel, [2]Clinical Biochemistry, Ben-Gurion University of the Negev, Beer-Sheva, Israel, [3]Biomedical Engineering, Johns Hopkins University, Baltimore, MD, USA, [4]Biology, Johns Hopkins University, Baltimore, MD, USA, [5]Chemistry, Ben-Gurion University of the Negev, Beer-Sheva, Israel.

S. cerevisiae Cin8p is a member of the kinesin-5 family of bipolar mitotic kinesins which are known to crosslink and slide antiparallel microtubules, thereby performing essential functions in spindle dynamics. Cin8p functionally overlaps with the homologous Kip1p during spindle assembly, metaphase and anaphase B and with dynein during anaphase B. Although Cin8p roles in spindle assembly and metaphase are well characterized, little is known about Cin8p functional overlap with Kip1p and dynein during anaphase B and Cin8p regulation during mitosis. To address this issue, we used VE-DIC microscopy to study the motor activity of biotinated Cin8p extracted from crude cell lysates on an avidin-coated glass surface. In addition, by real-time and immunostaining analyses, we examined *S. cerevisiae* anaphase B progression in cells which are deleted for Kip1p or dynein function, and express motor-domain mutants of Cin8p. Our results indicate that Cin8p phosphorylation and its ability to bind microtubules *in vitro* change during mitosis. In anaphase, Cin8p is phosphorylated and its microtubule binding is decreased, indicating that phosphorylation may regulate the motor activity of Cin8p. Our *in vivo* study revealed that while Cin8p overlaps with Kip1p in the fast and the slow phases of anaphase B spindle elongation, Cin8p overlaps with dynein during the final slow phase. Finally, we found that in cells in which spindle stability is compromised by a combination of *KIP1* chromosomal deletion and microtubule-binding-defected mutation of Cin8p, anaphase B spindle elongation is significantly delayed compared to wild-type cells. This delay is caused by a decrease in spindle elongation rate and by the existence of a checkpoint-like mid-anaphase pause between the fast and the slow anaphase B phases. Based on these results, we present a model for the mechanism by which Cin8p performs its anaphase roles.

Regulation of myosin V at the single-molecule level

Hailong Lu, Elena B. Krementsova, Kathleen M. Trybus.
Dept. of Molecular physiology and biophysics, University of Vermont, Burlington, VT, USA.

Myosin V (myoV) is a processive motor capable of moving multiple steps along an actin filament before dissociation, which suits it for its cellular role of organelle transport. However, the mechanisms by which myoV is recruited and its activity regulated *in vivo* are largely unknown. Earlier *in vitro* studies showed that the ATPase activity of the full-length myoV molecule is inhibited in the absence of calcium, and this inhibition is coupled to a conformational change to a compact conformation. We investigated the effect of calcium on processive run lengths of YFP-labeled myoV molecules, using TIRF (total internal reflection fluorescence) microscopy. Our results suggest that the characteristic run length of myoV is tightly controlled by the calcium and calmodulin concentration in solution. The data indicate that a calcium-dependent dissociation of calmodulin from myoV terminates its processive run. We also found that the activation and termination of processive runs by myoV may be accomplished by multiple mechanisms. We are currently investigating the interaction of myoV with the microtubule-based motor kinesin at the single molecule level.

The insertion L5 loop in human Eg5: is there a switch III in Kinesin-5 motor proteins?

Sunyoung Kim[1], Edward J. Wojcik[1], Richard A. Walker[2].
[1]Biochemistry & Molecular Biology, LSU Health Sciences Center, New Orleans, LA, USA, [2]Biological Sciences, Virginia Polytechnic Institute & State University, Blacksburg, VA, USA.

Originally defined by sequence conservation, switch regions in motor proteins are required for nucleotide binding and are hypothesized to initiate local and distal conformational changes upon selective interactions with appropriate substrates. An emerging prerequisite is that residues in switch sequences participate in salt-bridge interactions that serve as ‘gates’ during nucleotide hydrolysis. We note that proteins exploit conformational switches to modulate function not only by creating mechanical force, but also by controlling access to active sites and generating cooperativity. Thus, the definition of ‘switches’ can be broadened to include polypeptide segments capable of conformational switching. The discovery of small-compound inhibitors of Kinesin-5 proteins, like human Eg5, argues that extracellular loops in the motor domain may also provide functional specification for individual kinesin molecules and are indispensable for controlling cellular function. We hypothesize that the L5 loop is ‘switch III’ for the Kinesin-5 motor proteins, and identify hinge and intermediate regions of the loop, critical for allosteric function by structural and computational analyses. Toward testing this conceptual framework, difference FT-IR spectroscopy, with biochemistry and molecular biology, measures dynamics of protein movement and experimentally appraises chemical steps impacted by artificial, chemical regulators during mechanotransduction. Our biochemical and spectroscopic experiments argue that more than one salt bridge in Eg5 serve as ‘gates’ during ATP hydrolysis. As the L5 loop provides a member of this second ion pair, this extracellular loop can provide allosteric, biochemical tuning of human Eg5 activity.

Structure of Human Kip3d - Unconventional Microtubule Depolymerizing Kinesin

Katjusa Brejc, Lisa Belmont, Ming Yu, Yan Lee, Jim Hartman, Jun Guo, Serge Lichtsteiner, Kenneth Wood, Roman Sakowicz.
Cytokinetics Inc, South San Francisco, CA, USA.

Kip3 family members in yeast and *D. melanogaster* play an important role in the regulation of microtubule polymerization dynamics, but a mechanistic understanding of their effects on microtubules is not known. The human genome contains three kinesins that belong to Kip3 family. In this study, we present an enzymatic and structural characterization of human Kip3d. The crystal structure of the Kip3d motor domain was solved at a 2.2Å resolution. Despite overall similarity to other kinesin structures, Kip3d shows interesting differences, most notably in the position of β-sheet 5/α-helix 3 region relative to the core of the protein. The Kip3d structure has been determined in complex with Mg2+ADP, however the Switch I region and helix 4, the relay helix, adopt an 'ATP-like' conformation. Recently, two structures of microtubule depolymerization kinesins belonging to KinI family have been reported. There is no clear sequence similarity between Kip3 and KinI kinesins and yet these families share a similar function. We conclude that the Kip3 family kinesins represent a structurally unique family of microtubule depolymerizers.

Structural Basis for the Regulation of Myosin V

James R. Sellers, Ph.D.[1], Takeshi Sakamoto, Ph.D.[1], John A. Hammer, Ph.D.[2], Kavitha Thirumurugan, Ph.D.[3], Peter J. Knight, Ph.D.[3].
[1]Laboratory of Molecular Physiology, National Heart, Lung and Blood Institute, Bethesda, MD, USA, [2]Laboratory of Cell Biology, National Heart, Lung and Blood Institute, Bethesda, MD, USA, [3]Institute of Molecular and Cellular Biology, University of Leeds, Leeds, United Kingdom.

Structural Basis of the Regulation of Myosin V
Myosin 5 is a two-headed processive motor that translocates cargoes by taking 36nm strides along actin filaments. Its tail ends in paired globular tail domains (GTDs) thought to bind cargo. At nanomolar calcium, actin-activated ATPase is low and the molecule is folded. Micromolar calcium activates ATPase and the molecule unfolds. We now describe the structure of folded myosin in ATP and the GTD's role in regulating enzymatic activity. Electron microscopy of the folded myosins in the absence of calcium shows the two heads lie on either side of the tail and contact the GTDs. The lobe of the motor domain (Pro117 to Pro137) contacting the GTD contains conserved acidic side chains, suggesting ionic bonds stabilise the interaction. Myosin 5 heavy meromyosin (HMM), a constitutively active, two-headed fragment lacking the GTD, is inhibited by a dimeric GST-GTD fusion protein in the absence of calcium yielding a folded structure like intact myosin. Motility assays reveal that at nanomolar calcium HMM moves robustly on actin filaments in contrast to myosin where many fewer movements occur and few non-productive interactions occur. These results combine to show that in the absence of cargo, the GTDs of myosin 5 bind in a intramolecular manner to the motor domains producing a compact and inhibited structure that binds weakly to actin and allows the molecule to diffuse towards new cargoes.

Mechanism of regulation of myosin-I by calcium and calmodulin

John H. Lewis, Joseph M. Laakso, Tianming Lin, **E Michael Ostap**.
Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA.

Myo1b is a widely expressed myosin-I isoform that concentrates on endosomal and ruffling membranes and is thought to play roles in membrane trafficking and dynamics. Myo1b is alternatively spliced within the regulatory domain of the molecule, yielding isoforms with six ($myo1b^a$), five ($myo1b^b$) or four ($myo1b^c$) non-identical IQ motifs. We expressed the alternatively spliced myo1b isoforms truncated after the final IQ motif and included a biotinylation sequence at their C-termini to allow specific attachment of the myosin to motility surfaces via a biotin-streptavidin linkage. We measured the ATPase and motile properties of the recombinant myo1b splice isoforms at 37° C in the absence and presence of calcium, and we correlated these properties with calmodulin binding. We confirmed that calcium-dependent changes in the ATPase activity are due to calcium binding to the calmodulin closest to the motor, and we found that the primary effect of calcium is to accelerate the rate of phosphate release from actin-bound myosin. Sedimentation experiments show that calmodulin binds tightly to some of the IQ motifs ($K_d < 0.2$ μM) and very weakly to the others ($K_d > 5$ μM), suggesting that a subset of the IQ motifs are not calmodulin-bound under physiological conditions. We found the *in vitro* motility rate to be dependent on the myo1b isoform and the calmodulin concentration, and that the myo1b regulatory domain acts a rigid lever arm upon calmodulin binding to the high affinity and low affinity IQ motifs. Finally, we determined the rate at which calcium binds calmodulin and affects the rate of phosphate release.

Molecular Interaction Techniques for Investigating Molecular Motor Interactions with Tubulin and Anti-mitotic Drugs.

John J. Correia, PhD[1], P. Holland Alday, BS[1], Tom Laue, PhD[2], Walter F. Stafford, PhD[3].
[1]Biochemistry, Univ. Miss. Medical Center, Jackson, MS, USA, [2]Biochemistry and Molecular Biology, Univ. New Hampshire, Durham, NH, USA, [3]Analytical Ultracentrifugation Research Laboratory, Boston Biomedical Research Institute, Watertown, MA, USA.

The Analytical Ultracentrifuge (AUC) is extremely useful in characterizing molecular weight, hydrodynamic shape and molecular interactions. The combined use of (XLA) absorbance and (AU FDS) fluorescence optics introduces the advantage of specificity, sensitivity, selectivity and thus the ability to monitor sedimentation in complex molecular mixtures over an extremely wide concentration range. The powerful software package Sedanal offers the ability to fit globally multi-signal AUC data to complex hetero-reaction mechanisms. These rigorous approaches are highly appropriate for investigating the energetics and cooperativity exhibited in ternary complexes between tubulin, molecular motors and anti-mitotic drugs. Procedures and examples will be presented. (Supported by Biomolecular Interaction Technologies Center, UNH)

The Human kinesin Eg5 as a potential target for drug development

Frank Kozielski, **Dr**..
Laboratoire des Moteurs Moléculaires, Institut de Biologie Structurale, Grenoble, France.

Some mitotic kinesins represent potential drug targets for anti-cancer chemotherapy. Human Eg5 (kinesin-5 family) plays an essential role in establishing a bipolar spindle. Inhibitors of different chemical classes have been identified that target Eg5 and subsequently arrest cells in mitosis.
We identified S-trityl-L-cysteine (STLC) as a reversible, tight-binding inhibitor of Eg5 [1]. STLC inhibits basal and MT-activated ATPase activities as well as Eg5-driven MT sliding velocity, and slows MantADP release [2]. STLC binds to Eg5 in a pocket formed by a2 - L5 and a3 [3].
Subsequent cell-based assays revealed that STLC inhibits both separation of the duplicated centrosomes and bipolar spindle formation, thereby blocking cells specifically in the M phase of the cell cycle with characteristic monoastral spindles. Using live cell imaging, we observed prolonged mitotic arrest and subsequent cell death after incubation of HeLa cells with STLC. Immunoblot analysis of PARP-1, TRAIL, caspase-3, 8, and 9, and proteome analysis showed that STLC induced cell death predominantly by activation of the intrinsic apoptotic pathway [4].
Eleven residues in the inhibitor-binding pocket were mutated, to determine the key residues crucial for inhibition [5]. Leu214, was found to be essential for inhibition by STLC. Tumour cells might develop resistance to Eg5 inhibitors, by expressing Eg5 point mutants that retain the enzyme activity, but prevent inhibition, a feature that is observed for certain tubulin inhibitors.
1. DeBonis, S., et al., (2004). Mol. Cancer Ther. 3, 1079-1090. 2. Skoufias, D.A., et al., (2006). J. Biol. Chem. 281, 17559-17569. 3. Brier, S., et al.,. (2004). Biochemistry 43, 13072-13082. 4. Kozielski, F., et al., in revision. 5. Brier, S., et al., (2006). J. Mol. Biol. 360, 360-376.

Biosynthetic Tools to Investigate Cooperativity in Biomotor-Protein Assemblies

Michael Richard Diehl.
Bioengineering, Rice University, Houston, TX, USA.

The cellular mechanics of linear biomotor proteins often involves the cooperation of teams of motor proteins in the form of architecturally rich assemblies. Functioning in multiunit groups provides a means to regulate and enhance transport despite the stochastic behavior exhibited by single motor elements. Here, we explore such effects through the engineering of multi-biomotor protein assemblies, where the molecular properties of the assembly are precisely controlled. Our approach involves the design and synthesis of polymeric scaffolds that can be used as a backbone to assemble motors. Polymers are synthesized through the *in vivo* expression of artificial proteins. This method provides genetic level control over macromolecular architecture, and consequently, discrete control over number of coupled motors, intermotor distance as well as the nature of the elastic interconnects between motors. We find that organizing multiple motor proteins along a single backbone results in a dramatic enhancement of both the maximal ATP hydrolysis rate and the motor-complex velocity. Furthermore, coupling several proteins together removes the strain dependence of the velocity dependent step at zero load. These results demonstrate that our molecular handles over biomotor communication can be used to tune the local correlations between motors and to redirect the dynamical properties of an assembly. In this way, the materials approach adopted here can provide a novel means to deconvolve the molecular details that link the properties and function of multiprotein assemblies.

Properties of multiple-motor based transport and avenues for its regulation

Michael D. Vershinin, PhD[1], Brian C. Carter, MS[1], David S. Razafsky[2], Stephen J. King, PhD[2], Steven P. Gross, PhD[1].
[1]Developmental and Cell Biology, UC Irvine, Irvine, CA, USA, [2]School of Biological Sciences, University of Missouri - Kansas City, Kansas City, MO, USA.

Cargo transport in living cells is a tightly regulated process. Controlling the number of motors that, instantaneously or on average, move a cargo is an important potential avenue for such regulation. We explored this possibility in an in vitro bead assay. We observed that cargoes driven by multiple kinesin motors on bare microtubules (MTs) typically keep moving processively until reaching the end of the microtubule (exceeding ~8 micron of travel). This stands in sharp contrast to single motor driven beads, whose average processivity is on the order of one micron. We then repeated the experiments in assays where the MTs were incubated with varying amounts of the tau protein (a microtubule-associated protein known to affect MT-dynamics and axonal transport). The presence of tau on MTs was observed to depress cargo persistence, with by far the most prominent effect observed for multiple-motor driven beads. We propose a model of multiple-motor based transport consistent with our observations and explore the nature of tau's activity. We propose that tau does not merely block MT tracks but rather reduces the number of active motors by preventing the motors on the cargo from binding and rebinding to the MTs. This mechanism is analogous to the way tropomyosin regulates myosin access to actin filaments, however the effect of tau can be highly localized. Our data and modeling suggest novel ways in which transport can be regulated and disrupted locally. In the specific case of neurons, proper spatial distribution of tau can potentially aid efficient well-balanced transport. Conversely, our results show that misregulation of tau could disrupt axonal transport even when MT-dynamics is only weakly affected. Our results therefore provide new insight into the processes behind many neurodegenerative diseases such as the Alzheimer's disease.

Phosphorylation-induced Structural Changes in Smooth Muscle Regulatory Light Chain

David Kast J. Kast, L. Michel Espinoza-Fonseca, Sarah E. Blakely, David D. Thomas.
Physics, University of Minnesota, Minneapolis, MN, USA.

We have used site-directed fluorescent labeling, FRET, and molecular dynamics simulations to examine structural changes in the regulatory light chains of smooth muscle heavy meromyosin (HMM). Smooth muscle myosins are activated through phosphorylation of Ser 19 on the regulatory light chain (RLC). Our previous EPR experiments (Nelson et al., 2005) have shown that phosphorylation induces a disorder-to-order transition within the N-terminal phosphorylation domain of the RLC, accompanied by increased solvent accessibility, suggesting decreased head-head interactions. To test these hypotheses more directly, we have used molecular dynamics simulations to investigate the disorder-to-order transition and FRET to determine the separation of the RLCs. Simulations were performed on phosphorylated and unphosphorylated 25-residue N-terminal fragment of the smooth muscle regulatory light chain. After the first nanosecond, the residues K12-Q15 of the unphosphorylated fragment, remains disordered. In contrast, the phosphorylated fragment maintains strong α-helicity over the same residues during the entire simulation, indicating a disorder-to-order transition upon phosphorylation. In the fluorescence studies we labeled wild type and expressed mutant chicken gizzard RLC with donor and acceptor probes, and exchanged them onto HMM. Steady-state and time-resolved fluorescence demonstrates a decrease in resonance energy transfer, indicating the RLCs separate upon phosphorylation. The molecular dynamics simulations and fluorescence studies collaborates well with our previous EPR studies, demonstrating that phosphorylation induces both a disorder-to-order transition as well as separation between RLCs of the dimer.

Regulation of Kinesin-1 by Folding

David Hackney.
Carnegie Mellon Univ., Pittsburgh, PA, USA.

Conventional kinesin-1 undergoes autoinhibition through binding of a domain in the tail near the C-terminus to the motor domains at the N-terminus. In the absence of inhibition by tail domains, motor domains with bound ADP can bind to microtubules (MTs) with rapid release of ADP to form the tight rigor complex of nucleotide-free heads with MTs. Binding of tail domains inhibits the stimulation of ADP release by microtubules (MTs) and prevents formation of the tight rigor complex (Nature Cell Biol. 2, 257 (2000)). Tails not only inhibit the binding of heads to MTs, but also inhibit the basal release of ADP in the absence of MTs and thus act as general nucleotide release inhibitors. An additional factor is that tail domains contain an auxiliary MT binding site that can greatly increase the net affinity of kinesin for MTs by providing an additional interaction beyond that of the motor domains alone. Binding of head domains to tails produces a reciprocal masking of the auxiliary MT binding site in the tail and this contributes to the reduced MT affinity of the folded conformation. Recent work indicates that intermediate truncations from the C-terminus can result in unmasking of the auxiliary MT binding site, while still maintaining inhibition of ADP release in the absence of MTs. Current work is directed at dissecting the separate contributions to activation of the folded species from removal of the ADP inhibition by the tails and the unmasking of the auxiliary MT binding site. An additional important aspect is the influence of the binding of cargo or regulatory proteins on the inhibition by folding. The principal cargo binding site in the tail of the heavy chain is separate from the site that interacts with the heads. At least some cargo proteins that bind to this site do not relieve the inhibition of folded kinesin. Detailed studies of the relative affinities of the different binding partners will be required to determine if this is due to inability of the cargos to compete with heads for tail binding, or if the ternary complex of motors, tails and cargo is still inhibited. Regulation is likely to be the consequence of an interplay of factors that influence the binding of the tails to the heads, the exposure of the auxiliary MT binding site in the tail and post translational modification of the motor or cargo.

Do 2 or 3 Kinesin Motors Pull A Single Load Faster Than 1 Motor?

David B. Hill[1,2], **George Holzwarth**[1].
[1]Physics, Wake Forest University, Winston-Salem, NC, USA, [2]Cystic Fibrosis Center, University of North Carolina, Chapel Hill, NC, USA.

Although the mechanical behavior of molecular motors has been carefully studied in buffer, the mechanical behavior of motor-driven vesicles in cells is much less understood. We have tracked single vesicles in PC12 neurites with a spatial precision of ±30 nm and a time resolution of 120 ms. The velocity of individual anterograde vesicles was in most cases constant for periods of 1-2 s, then changed in a step-like fashion to a new constant velocity. We interpret the observed velocity steps as changes of ±1 and occasionally ±2 in the number of motors dragging that vesicle along a microtubule. In order to estimate the forces required to move the vesicle, we also measured the Brownian motion of unattached vesicles of similar size, again in live PC12 cells. From a Generalized Stokes-Einstein analysis of the Brownian motion, we determined the viscoelastic modulus of the PC 12 medium as experienced the vesicles. From this modulus and Stokes' Law, the drag force for vesicles of radius 0.3-0.4 μm found to be 4.2 ±0.6 pN at the lowest sustained velocity, presumably 1 motor. This is comparable to forces measured for kinesin in vitro. This work has been published [Eur. Biophys. J. 33, 623-632 (2004)].
The assertion that multiple motors pull a single cargo in vivo more quickly than a single motor can, remains controversial [Howard et al, Nature 342, 154-158(1989)]. To address this controversy, we are determining velocity-force curves when of 1, 2, and 3 full-length Drosophila kinesin heavy chain dimers pull a single load in vitro against a measured force.

Dynamic Cortical Rearrangements, Mechanosensing, and Mechanical Feedback of Living Cells

Douglas N. Robinson, Ph.D..
Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

Cell cortices rearrange dynamically to complete cytokinesis, crawl in response to chemoattractant, build tissues and make neuronal connections in environments where they are subjected to mechanical disturbances such as from neighboring cells or tissues. Highly enriched in the cortex, actin, myosin-II, and actin crosslinkers facilitate cortical movements. On the molecular scale, myosin-II generates mechanical force and senses mechanical loads, which alters its mechanochemistry. However, in the context of a whole nonmuscle cell where myosin-II acts on a complex, randomly arrayed cytoskeletal network, it is not known how myosin-II drives cell shape change, while promoting mechanical resistance. First, we used high resolution laser-based particle tracking (LPT) to probe the nanoscale, nonequilibrium cortical properties of living cells. From LPT studies, myosin-II mechanochemistry and dynacortin-mediated actin crosslinking control cortex dynamics in *Dictyostelium*. Consistent with its low duty-ratio, myosin-II does not directly drive active motility on long time-scales. Instead, myosin-II and dynacortin antagonistically regulate other active processes in the living cortex. Second, we discovered a novel mechanosensory and mechanical feedback system that is dependent upon myosin-II and that ensures robust cytokinesis. Mitotic cells sense and respond to applied forces by redistributing contractile ring proteins, including myosin-II, to the site of the mechanical disturbance, rejecting the disturbance, and completing symmetric cytokinesis. Our observations indicate that the spatial and temporal changes of cell shape during cytokinesis are controlled by mechanical feedback that overrides normal spindle signals and that directs the contractile ring apparatus anywhere along the cortex. This feedback system may provide critical regulation that ensures successful shape changes during cell division. Our combined effort is revealing the interface between myosin-II-driven active cortical rearrangements and passive cortical properties from actin crosslinkers that govern cell shape dynamics.

Two Point Mutations Near the Myosin Nucleotide-Binding Pocket Differentially Affect ATPase, Actin Motility, Muscle Structure and Physiology

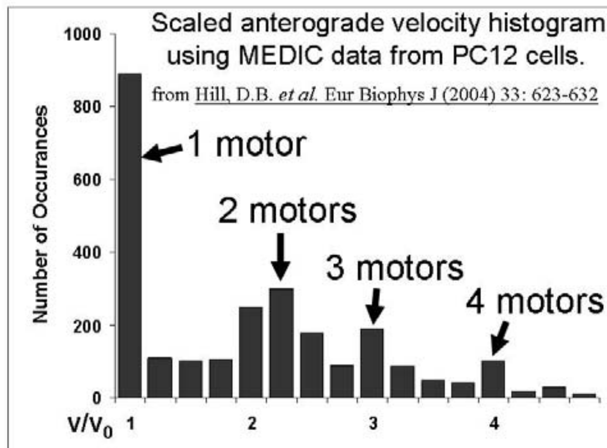
Corey M. Dambacher, B.S.[1], Anthony Cammarato, Ph.D.[1,2], Karen Occor, Ph.D.[2], Aileen F. Knowles, Ph.D.[3], William A. Kronert, B.S.[1], Rolf Bodmer, Ph.D.[2], **Sanford I. Bernstein, Ph.D.**[1].
[1]Biology, San Diego State University, San Diego, CA, USA, [2]Burnham Institute, La Jolla, CA, USA, [3]Chemistry, San Diego State University, San Diego, CA, USA.

We investigated the *in vivo* properties of two homozygous-viable *Drosophila melanogaster* myosin heavy chain mutants, *D45* (A261T) and *Mhc[5]* (G200D), and studied the *in vitro* properties of their mutated myosins. The *D45* mutation changes a residue near β7, while the *Mhc[5]* mutation is located at the base of loop 1. Both regions likely regulate the rate of nucleotide exchange. Ultrastructural analysis of indirect flight muscles (IFM) expressing *D45* myosin revealed wild-type myofibril structure, while flies expressing *Mhc[5]* myosin display severe hypercontraction by two days post-eclosion. *D45* myosin has decreased Ca^{2+} ATPase activity and reduced basal and actin-stimulated Mg^{2+} ATPase activity as compared to the wild-type IFM isoform (IFI). *Mhc[5]* myosin shows a greater than two-fold increase in basal Mg^{2+} ATPase activity but a similar actin-stimulated Mg^{2+} ATPase rate as the IFI. The average actin sliding velocity was reduced by two-fold for *D45* myosin compared to the IFI, while *Mhc[5]* myosin increased actin filament velocity by 15%. We investigated the effects of these mutations on *Drosophila* cardiac structure and performance. Beating hearts of semi-intact flies were imaged using direct immersion DIC optics in conjunction with a high-speed digital camera. Movies processed to produce edge tracings allowed us to monitor heart tube movements. Compared to wild type, *D45* hearts appeared dilated while *Mhc[5]* hearts exhibited non-contractile regions and apparently reduced chamber volume. Semi-automated computational analysis of cardiac performance showed perturbations in systolic and diastolic function for both mutants as well as alterations in rhythmicity for *Mhc[5]*. Overall, our results suggest that the less-active *D45* MHC yields normal IFM structure, but results in a dilated cardiac phenotype. In contrast, expression of the overactive *Mhc[5]* isoform results in IFM hypercontraction as well as cardiac contractile abnormalities.

Quantized intracellular transport velocities: discrete numbers of kinesin and dynein?

Jed C. Macosko, Ph.D., George Holzwarth, Ph.D..
Physics, Wake Forest, Winston-Salem, NC, USA.

Since 1975, histograms of intracellular transport have revealed quantized peaks, but only recently have these peaks been linked to discrete numbers of molecular motors working cooperatively. If true, how then do motors cooperate? Could cooperation explain higher intracellular velocities relative to extracellular results? To answer these questions, high resolution velocity data need to be examined. Motion enhanced differential interference contrast (MEDIC) microscopy can provide these data from any neuronal or neuron-like cell without requiring fluorescent labeling. MEDIC data from chick motoneurons, PC12 and honeybee Kenyon cells indicate that the quantized peaks are not an artifact and make it unlikely that different motors' velocities are integer multiples by coincidence. Instead these data suggest a link between numbers of motors and the quantized peaks. A model that combines kinesin force-velocity curves with Stokes' Law qualitatively fits the observed peaks. Quantitatively, however, kinesin force-velocity curves obtained from extracellular experiments do not fit the observed peaks. One possibility is that intracellular force-velocity curves are qualitatively similar to extracellular results, but are scaled by some factor. This scaling may derive from the interplay between kinesin and cofactors, which may explain higher velocities of intracellular transport.



X-ray interference studies of the configuration of the myosin heads during steady shortening of muscle.

Hugh E. Huxley, **Sc.D**.
rosenstiel, brandeis univ., waltham, MA, USA.

The characteristics of the interference fringes on the M3 and M6 reflections during isometric contraction and quick releases show that the actin-attached crossbridges are positioned in isometric contraction with their lever arm angles either in an approximately uniform distribution of ± 20°-25°, or possibly Gaussian of ±17°, around a position about 60° away from the rigor position seen in electron micrographs. In quick-releases few crossbridges have time to detach and the center of the same distribution shifts progressively nearer to rigor in releases up to 10nm.
We have now studied the corresponding behavior during steady shortening at loads of 0.9Po, 0.6Po, 0.5Po and 0.3Po. During slow shortening at the highest loads (0.9Po) the lever arm angles do not become dispersed through the whole range seen in quick releases, but merely shift the center of their distribution by about 7° towards the rigor position, with little or no change in the width of the distribution. At higher velocities of shortening (at 0.58Po, 0.5Po and 0.3Po) progressively larger shifts (of approximately 28°, 35° and 38°) are seen in the mean angle of the distributions, accompanied by increases in their widths. The data can be accounted for equally well by either uniform or Gaussian distributions of lever arm angles, but we have reasons to believe that uniform distributions are more likely. In that case, the heads will be distributed throughout most of what is taken to be the working stroke, about 10nm long, except at very low velocities of shortening, when they detach rather early in the stroke.
On the other hand, the X-ray data only give information on the positions of the myosin heads; not all the population attached to actin are necessarily developing active force.

Myo1c Binds Phosphoinositides through a Putative PH Domain

David E. Hokanson, **Joseph M. Laakso**, Tianming Lin, E Michael Ostap.
Physiology, University of Pennsylvania Sch Med, Philadephia, PA, USA.

Myo1c is a member of the myosin superfamily that binds PIP2, links the actin cytoskeleton to cellular membranes, and plays roles in mechano-signal transduction and membrane trafficking. We located and characterized two distinct membrane binding sites within the regulatory and tail domains of this myosin. By sequence and secondary structure analysis, we identified a phosphoinositide binding site in the tail to be a putative pleckstrin homology (PH) domain. Point mutations of residues known to be essential for phosphoinositide binding in previously characterized PH domains inhibit myo1c binding to PIP_2 *in vitro* and disrupt membrane binding *in vivo*. The extended sequence of this binding site is conserved within other myosin-I isoforms, suggesting they too contain this putative PH domain. We also characterized a previously identified membrane binding site within the IQ motifs in the regulatory domain. This region is not phosphoinositide specific, but binds anionic phospholipids in a calcium dependent manner. However, this site is not essential for *in vivo* membrane binding.

A Microtubule-destabilizing Kinesin-13 Motor in Meiosis

Jianwei Zou, PhD, Christine D. Yankel, MS, Kevin Su, **Sharyn A**. **Endow**, **PhD**.
Dept Cell Biol, Duke Univ Med Ctr, Durham, NC, USA.

The Kinesin-13, Klp10A, is a microtubule depolymerizer in *Drosophila* that regulates microtubule dynamics in mitosis. Klp10A binds to mitotic spindle poles and centromeres, and depolymerizes microtubule minus ends, promoting poleward flux - slow poleward movement of spindle microtubules. During interphase, Klp10A is targeted to microtubule plus ends by EB1, where it reportedly stimulates catastrophes, growth-to-shrinkage transitions. Up to now, little is known about Kinesin-13 proteins in meiosis. Here we show that full-length Klp10A-GFP is associated with the pole bodies of anastral oocyte meiosis I spindles, and decorates meiotic chromosome centromeres and oocyte cortical microtubules. The pole bodies of meiosis I spindles can be observed bound to fluorescently labeled cortical microtubules in *klp10A-gfp* oocytes, indicating that Klp10A binding both to the pole bodies and cortical microtubules could anchor spindles to the cortex. Microtubule destabilization by colchicine resulted in rudimentary spindles that were displaced towards the oocyte interior, and ~2-fold longer Klp10A-GFP signals on cortical microtubules than controls. Microtubule stabilization by paclitaxel resulted in either elongated or foreshortened spindles that were displaced from the cortex and ~2-fold shorter Klp10A-GFP signals on cortical microtubules than controls. These results show that microtubule dynamics plays an important role in maintaining oocyte meiotic spindle structure and position, and indicate a role for Klp10A in cortical anchoring of spindles. They demonstrate that Klp10A binds with higher affinity to destabilized than stabilized microtubules in oocytes during meiosis, contrasting with a previous report that the motor targets to polymerizing, rather than depolymerizing microtubule plus ends during interphase.

In vivo measurement of molecular motor forces

George T. Shubeita, PhD, Steven P. Gross.
Developmental and Cell Biology, University of California, Irvine, Irvine, CA, USA.

The internal organization of living cells is crucial for their proper functioning. To achieve and maintain that organization molecular motor proteins move various cargos to different areas inside the cell. While i*n vitro* studies have been instrumental in our understanding of some basic properties of the function of different motors, much less is known about their function and the relevance of their mutual interactions (as well as interaction with other proteins) inside living cells. A key issue that is still unresolved is the number of motors moving a cargo in cells and how these motors work together. While there have been suggestions that cargoes are often moved by multiple motors, they were indirectly inferred from cargo velocity measurements. Those are not necessarily a good readout since they rely on an untested model of cargo transport. To move a cargo inside a cell motors need to exert force, hence measuring that force amounts to directly probing their function. To that end, we have constructed a specialized optical trap system capable of measuring the force that motors can exert while hauling *individual* lipid droplets along microtubules in *Drosophila* embryos. These measurements reveal that multiple motors work together *in vivo* and that same polarity motors cooperate in a dynamic fashion to haul lipid droplets. Moreover, commensurate peaks in histograms of forces show that only a few motors (1-5) move the lipid droplets. Changes in the number of motors and motor cooperation as a function of the net direction of transport will also be presented.

Structural Genomics of Human Kinesin Motors

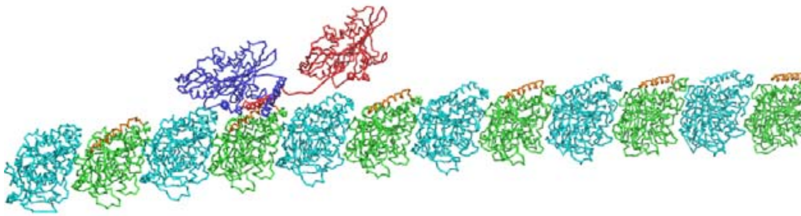
J Wang, W Tempel, Y Shen, L Shen, A Bochkarev, **Hee-Won Park**.
Structural Genomics Consortium, University of Toronto, Toronto, ON, Canada.

We, part of Structural Genomics Consortium, have a goal to solve the structures of human kinesin proteins, if not all but at least one structure for each subfamily. These collective human KIF structures provide a basis for understanding how kinesin motor works. We have implemented high throughput procedures for all steps from cloning to structure calculation. In this poster, we describe the structures of KIF2 and KIFC3 motor domains and the structure of forkhead-associated (FHA) domain of KIF1C. First, KIF2 is a member of kinesin-13 family (MCAK/KIF2), which is known to depolymerize microtubules. The human KIF2 motor domain resembles the previous mouse KIF2 motor domain, including the long helical neck and the finger-like loop L2. This finding suggests a common mechanism for microtubule depolimerization by human and mouse KIF2. Second, KIFC3 is a member of kinesin-14 (C-terminal motor), which is a microtubule minus end-directed motor. The human KIFC3 motor domain is structurally similar to the previous kinesin-3 members (ncd and kar3). Based on this structural similarity, we hypothesize that the stalk rotation of human KIFC3 constitutes a force-producing stroke that propels the motor toward the minus end. Third, KIF1C is a member of kinesin-3, which is implicated in Golig-ER transport and in mouse macrophage resistance to anthrax toxin. A recent finding suggests the role of human KIF1C in podosome regulation. The KIF1C FHA domain consists of nine beta strands connected by loops, similar to other known FHA domain structures. However, it is still unclear if the KIF1C FHA domain is involved in the catalytic cycle of ATP hydrolysis, or if it is a phosphorylation-dependent cargo docking site.

Modeling molecular motor: Two-headed kinesin study.

Changbong Hyeon, **PhD**, Jose Nelson Onuchic, PhD.
Center for theoretical biological physics, UC San Diego, La Jolla, CA, USA.

Based on the recent experimental propositions we designed a minimal coarse-grained model of two-headed kinesin (kinesin-I) on the protofilament of microtubule and performed Brownian dynamics simulations that enabled us to extract the important physics of molecular nanomachine. Kinesin head, neck linker, coiled-coil, and the binding sites along the protofilament are explicitly designed. We showed that the binding of the disordered neck linker to the kinesin head whose binding motif to the neck linker is oriented in one direction along the protofilament leads to the unidirectional movement of the kinesin molecule. Each step of the motion consists of power stroke and thermal ratchet motion. Our model is also useful in understanding, at molecular level, the effect of load on the velocity of processive motion. Comparison of average contact map between leading and tailing head reveals the role of strain built along the neck linker when both heads are bound on the microtubule. The strain through the neck linker leads to the opening of ATP binding site at the leading head, which suggests the facilitation of ATP binding. Visualization using molecular modeling sheds light on the working mechanism of a complex system.



Twirling of Myosins II, V and VI

John F. Beausang, M.S.[1], Yuijie Sun, PhD[2], Yale E. Goldman, MD, PhD[2].
[1]Phyiscs, University of Pennsylvania, Philadelphia, PA, USA, [2]Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, PA, USA.

Several factors are expected to determine whether actin experiences a torque during active myosin-directed sliding: the direction of the force vector between actin and myosin, the distribution of myosin binding sites on actin, and cooperation between myosins translocating an individual actin filament. Consequently, studies in the literature have disagreed on whether myosin moves axially along actin or helically with a right-handed or left-handed pitch. Different results are obtained from muscle myosin and various unconventional myosin isoforms. We have developed a new assay to monitor azimuthal rotation of actin in a gliding filament assay using polarized total internal reflectance fluorescence microscopy. The actin is sparsely labeled with tetramethylrhodamine at Cys^{374} and the three-dimensional orientation of the individual rhodamines is monitored during *in vitro* filament gliding with 40 ms time resolution. To determine the handedness of any twisting motions, extra ± 45° excitation polarizations were added to our earlier single molecule fluorescence polarization method (Forkey et al., *Nature* 422:399, 2003). During translocation by myosin, approximately half of the observed actin filaments exhibit a 'twirling' helical path of rotation around the filament axis. Myosin II and V consistently induce a left-handed twirling motion (opposite to the long-pitch helix of actin) with pitch 1.0 ± 0.2 µm and 1.5 ± 0.1 µm, respectively. Conversely, Myosin VI, which translocates actin in the opposite direction as myosins II and V, consistently induces a left-handed helical pitch of 1.2 ± 0.1 µm. The observed twirling motions may be the result of an applied torque between the two proteins that is required in some theories of actomyosin motility. Unlike myosin VI, neither myosin II nor myosin V follow the actin helix. Supported by NIH grant AR26846 and NSF grant DMR04-25780.

Walking Mechanism of Kinesin Studied by the Unbinding-Force Measurements

Rumiko Akiyama[1], Yusuke Oguchi[1], **Shin'ichi Ishiwata**[1,2].
[1]Department of Physics, Waseda University, Shinjuku-ku, Tokyo, Japan, [2]Advanced Research Institute for Science and Engineering, Waseda University, Shinjuku-ku, Tokyo, Japan.

Kinesin is a dimeric ATP-driven motor protein that moves processively along a microtubule. Its high processivity has been explained by a mechanism, which postulates the repetitive switching between single- and double-headed binding to a microtubule. It is thus essential to determine the binding manner and the strength of the kinesin-microtubule interaction in various nucleotide states. We have developed a microscopic technique using the optical tweezers to identify the binding manner in each nucleotide state (Kawaguchi, K. & Ishiwata, S., *Science* **291**, 667-9, 2001; Uemura, S., *et al*., *PNAS* **99**, 5977-81, 2002), and, in addition, to estimate the transition rate between single- and double-headed binding by measuring the unbinding-force distribution at various loading rates (Kawaguchi, K., *et al*., *Biophys. J*. **84**, 1103-13, 2003). We found that the external load imposed on a single native kinesin molecule during a single-headed binding in the direction of motility enhances its ADP affinity, whereas that imposed in the reverse direction decreases it (Uemura, S. & Ishiwata, S., *Nature Struct. Biol*. **10**, 308-11, 2003). Here we confirmed this property using a single-headed kinesin construct, and also examined the bond strength under several other conditions, such as the presence of ADP and Pi. We stress that such a loading direction-dependent mechanochemical coupling is consistent with the idea that the internal load imposed between the two heads plays a key role in regulating the ATPase cycle in each head, and ensures the unidirectional and cooperative movement of processive motors. Also, the effects of temperature on the kinesin motility, i.e., sliding velocity, duration of movement and run length (processivity), will be discussed (Kawaguchi, K. & Ishiwata, S., *BBRC* **272**, 895-9, 2000 & *Cell Motil. Cytoskel.* **49**, 41-7, 2001; Nara, I. & Ishiwata, S., *BIOPHYSICS*, **2**, 13-21, 2006).

A Myosin V Assay Using Linear Zero-mode Waveguides to Observe Differential Nucleotide Dynamics

L Stirling Churchman[1], Jonas Korlach[2], Mathieu Foquet[2], Michael J. Levene[3], James A. Spudich[4].
[1]Department of Physics, Stanford University, Stanford, CA, USA, [2]Pacific Biosciences, Menlo Park, CA, USA, [3]Department of Biomedical Engineering, Yale University, New Haven, CT, USA, [4]Department of Biochemistry, Stanford University, Stanford, CA, USA.

We previously developed a technique allowing for the simultaneous co-localization of two chromatically differing fluorophores called single-molecule high resolution co-localization (SHREC). We employed SHREC to measure the end-to-end distances of dsDNA and to directly observe myosin V molecules walking hand-over-hand. We are adapting the SHREC technique to observe myosin V's nucleotide dynamics using dye-labeled ATP molecules. The coordinated ATPase activity of myosin V has not been directly studied by single molecule techniques. Bulk biochemical studies of the myosin V dimer have been challenging and are limited. Single molecule fluorescence studies of myosin V with dye-labeled ATP would require micromolar concentrations of labeled ATP. The background caused by free fluorescent labels is substantial above a concentration of 10-30 nM when using total internal reflection fluorescence microscopy. Circular zero-mode waveguides have been used to increase this concentration to 7.5 uM to directly observe nucleotide incorporation by DNA polymerase (2). We are using linear zero-mode waveguides to study myosin V's mechanochemical cycle due to the long actin filaments necessary for the assay. We have fabricated linear zero-mode waveguides with a range of widths and are characterizing them computationally and experimentally. Using ~ 50 nm wide linear zero-mode waveguides we have increased the working concentration of labeled ATP to greater than 1 uM. We have developed an experimental geometry that places actin filaments along the bottom of the linear zero-mode waveguides and allows myosin V molecules to be observed while walking processively along them.

Plus End-specific Depolymerase Activity and Plus End-directed Motility of Kip3 (Kinesin-8) Explain Its Role in Positioning Mitotic Spindles During Asymmetric Cell Division

Mohan Gupta[1], Pedro Carvalho[1], David Roof[2], David Pellman[1].
[1]Dana-Farber Cancer Institute, Boston, MA, USA, [2]University of Pennsylvania, Philadelphia, PA, USA.

Budding yeast Kip3 is a member of a conserved family of microtubule motors (Kinesin-8) required for microtubule-cortical interactions, normal spindle assembly, and kinetochore dynamics. We demonstrate that Kip3 is both a plus end-directed motor and plus end-specific depolymerase, a unique combination of activities not found in other kinesins. The Kip3 ATPase activity was stimulated by both microtubules and unpolymerized tubulin. Furthermore, Kip3 in the ATP-bound state formed a complex with unpolymerized tubulin. Thus, Kinesin-8s may depolymerize microtubules by a mechanism that is similar to that used by non-motile Kinesin-13 proteins. Fluorescent speckle analysis established that in vivo Kip3 moved toward and accumulated on the plus ends of growing microtubules, suggesting that motor activity brings Kip3 to its site of action. Globally, and more dramatically upon cortical contact, Kip3 promoted catastrophes, pausing, and inhibited microtubule growth. These findings explain the role of Kip3 in positioning the mitotic spindle in budding yeast and possibly other processes controlled by Kinesin-8s.