2671-Pos Board B641

Direct Measurement of the Relative Contributions of Turgor Pressure, the Peptidoglycan Cell Wall and Cytoskeletal Filaments to Gram-negative Prokaryotic Cell Mechanics using AFM

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The envelope of a gram-negative bacterium is composed of three layers: the inner and outer membranes and the periplasmic peptidoglycan (PG) cell wall. The PG layer is thought to be the stress bearing structure that determines and maintains cell shape, preventing cells from bursting due to a large inner turgor pressure. In vitro, PG mechanics have been measured with different techniques, yielding a high elastic modulus. However, in vivo such mechanical measurements are complicated by the turgor pressure. On the other hand, direct measurement of a cell's turgor pressure is difficult due to the presence of the PG layer. We are able to overcome these difficulties and simultaneously measure cell turgor pressure and PG mechanics in vivo with an atomic force microscope (AFM). Using a vancomycin sensitive strain of Escherichia coli, we locally induced small membrane blebs at sites of reduced PG integrity. AFM indentation of these membrane-bound blebs directly probes the turgor pressure. By comparing bleb indentation with indentation of the cell body, we can deduce the relative contributions of the PG layer and turgor pressure to the overall cell mechanics. Furthermore, drugs that inhibit bacterial cytoskeletal filaments can be used to evaluate the role of these proteins in modulating cell elasticity.

2672-Pos Board B642

Mechanism of MSP-based Cell Body Retraction in the Amoeboid Sperm of Nematodes

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Cell body retraction in the amoeboid sperm *Ascaris suum* is generated by rearrangement and disassembly of the MSP cytoskeleton, without involvement of the motor proteins that pull the cell body forward in actin-based crawling cells (Miao et al. 2003. Science 302:1405). Reconstitution of retraction *in vitro*, which involves shortening of columnar meshworks of MSP filaments called fibers that assemble in cell-free extracts of sperm, has enabled us to explore the biochemical and biophysical basis of retraction. We found that fiber retraction is induced by treatment with low pH buffers with an optimum effect on both the rate and extent of retraction at pH 6.0. 3D correlative light and electron microscopy showed that there is a progressive loss of filament mass during retraction. However, the filament length distribution within fibers did not change significantly suggesting that retraction involves selective loss of filaments rather than uniform disassembly of all filaments in the fiber. Stereology revealed that during retraction filaments tended to rearrange from a random orientation to alignment along the long axis of fiber. (degree of orientation = 20 to 30%). Following the movement of small vesicles trapped filament mesh of the fiber, which appear as ''specks'' under optical microscopy, showed that shortening was faster and more extensive in the newest portion of the fiber. Moreover, we found that specks that started several microns apart could overtake one another but then moved in tandem as the fiber continued to shorten. These results suggest that the pattern of filament rearrangement is not uniform throughout the fiber. We are currently seeking to apply fluorescence speckle microscopy to explore the dynamics of filament rearrangement at higher resolution. Supported by NIH Grant R37 GM29994.

2673-Pos Board B643

FRAP Analysis Combined With A Single-cell Electroporation Technique In Sea-urchin Spermatozoa

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In sea-urchin spermatozoa, energy required for flagellar motility is provided by ATP diffusion from mitochondria located at the proximal ends of flagella along with the creatine shuttle system. However, no direct analysis of the diffusion rates inside flagella has been carried out thus far. Using a FRAP (fluorescence recovery after photobleaching) technique, we determined the diffusion coefficients of fluorescein-derivatives (AM esters of calcein, carboxyfluorescein, and Oregon Green; MW 376-623) to be 63-64 $\mu m^2/s$. Although these values are about one third of the estimates that were previously used for theoretical calculations (Tombes et al., 1987), we concluded that the rate of ATP diffusion inside spermatozoa was high enough to support the continuous motility of sea-urchin sperm flagella if creatine shuttle system is working. In order to investigate diffusion properties of fluorescent dyes of a wide range of molecular weights, we used a single-cell electroporation (SCE) technique, which has been developed for nerve cells (Bestman et al., 2006). Using the technique, we succeeded to inject fluorescein dextran of MW 3,000 (3k-FD) into sea-urchin sperm cells. By FRAP analysis, we determined the diffusion coefficients of 3k-FD to be ~30 $\mu m^2/s$, about one half of that of carboxyfluorescein (MW 376), almost consistent with the value estimated from the molecular weights. We also investigated the diffusion properties through the ''neck'' regions, between the head and tail of spermatozoa. When the head region of calcein-loaded spermatozoon was photobleached, slow recovery of head fluorescence along with the decrease of fluorescence signal in the tail region was observed. It suggests that small molecules like calcein (MW 623) can move almost freely through the boundary between the head and tail.

2674-Pos Board B644

System Analysis of the Ciliary Response to Red Light in *Chlamydomonas reinhardtii*

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To understand signal processing networks within cells we study the motile green alga *Chlamydomonas reinhardtii*. This organism swims with two cilia and has an eye with rhodopsin (peak sensitivity 500 nm, green) controlling the direction of its swimming (phototaxis). Here we report on a red light receptor (peak sensitivity 670 nm) that influences the frequency of ciliary beating, which we monitor by observing a single cell held on a micropipette with a quadrant photodiode. For square-wave on-off stimuli, the recorded beating frequency shows the on-step latency is strongly light intensity dependent, ranging from 700 ms at 1.3 W/m^2 to 200 ms at 50 W/m^2. This long delay suggests a diffusive step in the signaling pathway and contrasts with the sub-milliseconds latency of green light stimuli. The response amplitude is sustained for the full length of the on-step and is also light intensity dependent. However, the off-step latency seems light independent ranging from 400 to 1600 ms. Following the light off, the beating frequency drops more rapidly and farther the greater the prior light intensity. Products appear to be synthesized in proportion to the light intensity, but once the light is off these products in the cilium run out after about a second. Since the beating frequency is feedback regulated, the red-light elevation of the beating frequency is compensated by a counter molecular change in the cilia. Consequently, when these products run out, there is a rapid declined in beating frequency that takes about 10 s to recover. In addition to steps, sine and white-noise stimuli have been used to refine the response function.

2675-Pos Board B645

Abnormal Movement And The Trend Of Flagellar Force Production During Regeneration In *Chlamydomonas Reinhardtti*

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Several respiratory, digestive, and reproductive disorders result from dysfunctional cilia and flagella. Because the form, strength or frequency of flagella motion is abnormal in these disorders, biological functions performed by the cells utilizing flagella and cilia are debilitated. The causes of debilitation are not known as the internal mechanism for creating the flagella's breast stroke-like motion is still not fully understood. This study uses *Chlamydomonas reinhardtii* flagella as a model system and reports standardization of PSD calibration, laser trapping and cell movement recording methods. When motion in a laser trap is viewed as a trace, natural cell rotation while swimming (caused by flagellar dominance) results in a spirograph-like 'donut' shape. Circle fit analysis programs were written to measure trends in force generation during flagellar regeneration. The percentage of 'donuts' per flagella length increases linearly, similar to the increase in length over regeneration time. However the flagellar force trend shows a significant, unexpected dip during flagella lengthening. Neither the flagella infrastructure necessary to establish dominance nor the causes of the decrease in force are known, thus these data open many new research directions.

2676-Pos Board B646

How Does Stall Force Affect Contractions of a Biological Spring, *Vorticella convallaria?*

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The stalk of *Vorticella convallaria*, a sessile peritrich, is considered as a model biological spring for bio-inspired actuators because of its remarkable speed and force. When stimulated, the stalk of *Vorticella* contracts over a few hundreds micrometers in a few milliseconds, and its energy source is not ATP but

Ca^{2+}. Because major components of the spasmoneme, the contractile organelle inside the stalk, are EF-hand Ca^{2+}-binding proteins including spasmin and centrin, the spasmonemal contraction is thought to be related to other centrin-based motility mechanisms. This study describes how stall force affects contractions of live *Vorticella*. To impede contractions, we applied hydrodynamic drag force to *Vorticella* in a microfluidic channel with Poiseuille flow of viscous PVP solution. This method enables controlling the stall force by changing flow rate and the viscosity of the solution. Cell dimension measurements show that the zooid is elongated by the flow in relaxed and contracted states keeping roughly constant volume. As the stall force increases, the end-to-end length of the contracted stalk increases while that of the relaxed stalk is almost constant, and maximum contraction speed decreases while contractions take longer time. Furthermore, the time lag in contraction commencement between the zooid and the stalk also increases. We measured time differences in movement start among polystyrene beads attached to the stalk, and they increase with increasing stall force. These increasing time lags imply that the stalk cannot contract until it develops force great enough to overcome the stall force. The stall force affects the relaxation of *Vorticella* because relaxations take longer time as the stall force increase and the extending stalk resumes its contraction after the stall force is removed. It seems that although the spasmoneme retains contractile force, the stall force extends the stalk.

2677-Pos Board B647

Tuning Cellular Mechano-Response Using Biomembrane-Mimicking Substrates of Adjustable Fluidity

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An important aspect of mechanobiology is that tissue cells are anchorage-dependent and respond to viscoelastic changes in their environments. The mechanosensitivity of cells is believed to play an important role in processes such as cancer cell migration and stem cell differentiation. Previously, cellular mechano-response has been mainly studied using μm-thick polymeric films of adjustable viscoelasticity. Here we report on the design and characterization of alternative cell substrates based on 8-40nm thick polymer-tethered phospholipid membranes where cellular mechano-response can be regulated by tuning bilayer fluidity. Two complementary membrane systems are employed to span a wide range from low to high bilayer fluidity. Low to medium bilayer fluidity is achieved by using a single polymer-tethered lipid bilayer of adjustable tethering concentration. Medium to high bilayer fluidity is obtained through the regulation of the number of bilayers in a stack of polymer-tethered lipid bilayers. Changes in bilayer fluidity in these substrates have been confirmed through wide-field single molecule tracking of fluorescently labeled lipids. To facilitate the adsorption and migration of cells, these biomembrane-mimicking substrates contain bilayer-cell linkages of well-defined concentrations. Phase contrast microscopy experiments on PC12 neurons show that dendritic growth can be tuned by modifying the tethering concentration in a single polymer-tethered lipid bilayer. Comparing phase contrast and epifluorescence microscopy experiments on 3T3 fibroblasts containing GFP-actin, which were plated on multi-bilayer stacks, revealed profound changes in cellular phenotype, projected cell area, cell migration, and cytoskeletal organization with the number of bilayers in the stack. For example, on very fluid substrates, neuron-like, dendritic fibroblasts were observed. The described substrates are particularly significant because, unlike in the case of polymeric films, substrate-cell linkages are free to move and matrix remodulation caused by adsorbed cells is largely suppressed.

2678-Pos Board B648

Cell Contact, Substrate Mechanics And Boundary Conditions In The Movement Of Epithelial Sheets

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The directed and highly coordinated movement of epithelial cells can be found in various vertebrate systems, from the separation of tissues in early development through the renewal of tissues in the adult. How the cells coordinate their movement in a sheet remains unexplained, especially in physical terms, as the movement involves a complex balance of forces generated at multiple lengthscales. The forces that stem from the cellular level, and their mediation by the physical environment that allow them to manifest a highly correlated, multicellular pattern of movement is dependent upon the relationship between local influences such as cell-cell contact and the ability for cells to deform the substrate, with longer-ranged influences, such as physical and geometric constraints placed on the population of the sheet. Therefore, in this study, we quantitatively explore the dependence between cadherin-mediated contact, substrate mechanics, and boundary conditions on the motility of two-dimensional epithelial sheets.

2679-Pos Board B649

TβRIII Restores Normal Cytoskeleton Mechanics In Ovarian Cancer Cells

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Changes in cellular phenotypes in cancer are characterized by alterations of the cytoskeleton and several important signaling pathways in the cell. One of the signaling pathways implicated in controlling proliferation, angiogenesis and apoptosis is the TGF-β signaling pathway. Loss of expression of the TGF- β superfamily co-receptor, TβRIII/betaglycan, occurs in a broad spectrum of cancers, including those of the breast, ovary and prostate. Recent studies have shown that restoration of TβRIII to metastatic populations of ovarian and breast cancer cells suppresses migration via alteration of the cytoskeleton. Using our 3 dimensional force microscope system (3DFM) for passive and active microrheology, we compared the response of normal ovarian surface epithelial cells, ovarian cancer cells with reduced TβRIII expression, and ovarian cancer cells with restored expression of TβRIII. We found that cancer cells lacking TβRIII were at least 3× more compliant (less stiff) than either normal epithelial or TβRIII-expressing cancer cells. Our results, are consistent with potential invasiveness being correlated with increased cell compliance, and both being regulated by the TβRIII pathway. Compliance measurements using the 3DFM could be a useful tool to measure invasiveness in the future.

2680-Pos Board B650

Three Dimensional Superresolution Fluorescence Microscopy Reveals Protein Stratification in Focal Adhesions

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Focal Adhesions (FA) are dynamic structures consisting of large numbers (>150) of different proteins that mechanically link the actin cytoskeleton to the extracellular matrix (ECM). Despite the central role of FA in cell migration and the wealth of biochemical and cell biological data on FA proteins, it remains virtually unknown how these proteins are organized within FA. Based on the differential dynamics of distinct FA proteins we previously observed using fluorescent speckle microscopy, we hypothesized that FA proteins may be organized into stratified layers within FA that serve as dissipative elements in a ''molecular clutch'' to form a regulatable, force-transducing link between the actin cytoskeleton and the ECM. To test this hypothesis, we employed a 3-dimensional superresolution fluorescence microscopy technique, interferometric photoactivated localization microscopy (iPALM), to determine sub-20 nm z-axis localizations of several key structural components of FA labeled with photoactivatable fluorescent proteins and expressed in U2OS cells plated on a fibronectin-coated substrate. Within FA, we found that the cytoplasmic face of the plasma membrane, marked by Farnesylated tdEosFP, was localized at ~20-30 nm from the substrate, in agreement with previous electron miocroscopic analyses. Talin and vinculin, putative force transducing elements of FA, were observed within distinct planes parallel to the substrate, with the highest densities at ~35, and ~50 nm above the substrate, respectively. Actin appeared with the highest density at ~90 nm above the substrate plane, and was largely excluded from area adjacent to plasma membrane up to a height of ~50 nm. iPALM reveals for the first time the organization of specific proteins within the nanoscale core of the FA. The protein stratifications in FA provide a structural context for the mechanosensing and mechanotransducing functions of FA.

2681-Pos Board B651

Role of Mechanotransduction in Cellular Processes

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The role of mechanical properties of cells is gaining increasing attention due to the regulatory role that it plays in cellular processes. In particular the transduction of an applied force on the cell membrane through the cellular components can have significant influence on such phenomena as stochasticity in gene expression and cancer metastasis. We are currently investigating the effect of mechanical forces on the stochasticity of gene expression in E.coli cells and