spectroscopy (FCS). FCS is an advanced microscopy technique in which fluctuations in the fluorescence of a dye or dye-labeled molecule is recorded as the particles freely diffuse through a small focal volume. In this case, we measured the fluorescence of dye-labeled (Nile Red) HDL in the presence of wild type and mutant EL. The data can be analyzed mathematically using the cross-correlation function, from which the diffusion coefficient of the molecule is obtained. The lipase activity of EL changes HDL size, in turn affecting the diffusion coefficient, and can be calculated using the Stokes-Einstein relation. Our preliminary results suggest the hydrolysis of HDL occurs rapidly and proportionately to the concentration of EL. Several mutations in EL have been identified in human population studies. Our future goal is to compare the rate of hydrolysis between wild type and mutant EL and with normal and oxidized HDL. In addition, fluorescence measurements were used to investigate the composition of HDL. The fluorescence spectrum of Nile Red is dependent upon the local lipid environment. By monitoring the change in fluorescence emission as a function of EL metabolism, we investigate the content of HDL while being remodeled by EL. These fluorescence techniques allow us to answer some of the key questions regarding the HDL lipid collection and distribution function.

3903-Pos

In Vivo Imaging of Single-Molecule Translocation through Nuclear Pore Complexes by Pair Correlation Functions

Francesco Cardarelli, Enrico Gratton.
University of California, Irvine, CA, USA.

Nuclear pore complexes (NPCs) mediate bidirectional transport of proteins, RNAs, and ribonucleoproteins across the double-membrane nuclear envelope. We recently introduced a method based on pair correlation functions (pCF) which measure the time the same molecule takes to migrate from one location to another within the cell (1). The spatial and temporal correlation among two arbitrary points in the cell can provide a map of molecular transport, and also highlight the presence of barriers to diffusion with very high time resolution (in the microsecond scale) and spatial resolution (limited by diffraction).

Here we report the use of this method to directly monitor a model protein substrate undergoing transport through NPCs in living cells, a biological problem in which SPT has given results that cannot be confirmed by traditional FCS measurements because of the lack of spatial resolution. Our substrate is composed by a GFP linked to a functional nuclear localization sequence (NLS) and transfected into living CHO-K1 cells: the recombinant NLS-GFP protein can bind to molecular carriers mediating cytoplasm-to-nucleus active import as well as shuttle across the NPC by passive diffusion (its molecular weight is below the cut-off size limit of the NPC).

We show that obstacles to molecular flow can be detected and that the pCF algorithm can recognize the heterogeneity of NLS-GFP intracompartment diffusion as well as the presence of barriers to its transport between compartments (i.e. the NPCs of the nuclear envelope).

(1) Digman, M.A., and Gratton, E. Imaging Barriers to Diffusion by Pair Correlation Functions . Biophys. J. 97, 665-673 (2009).

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3904-Pos

Fluorescence Correlation Spectroscopy for Clinical Testing in Von Willebrand Disease

Richard Torres, Michael Levene.
Yale University, New Haven, CT, USA.

The von Willebrand factor (vWF) protein is an essential component of normal coagulation that is present in human plasma as a distribution of multimers composed of 2 to 40 or more monomers. Defects in the synthesis and metabolism of vWF represent the most common inherited abnormalities of coagulation and can be categorized as type 1 for quantitative deficiencies and type 2 for qualitative deficiencies. Current clinical methods for diagnosis and classification of von Willebrand disease suffer from significant limitations relating to the vast number of mutations that can occur, the fact that multimer size is a critical determinant of functional capacity, and the poor reproducibility of available activity assays. We have successfully employed the use of fluorescence correlation spectroscopy (FCS) to address the drawbacks of presently available vWF analysis methods. Autocorrelation curves from fluorescently tagged anti-vWF antibody incubated with plasma from normal donors and controls differ significantly from those obtained with plasma from patients with von Willebrand disease. Furthermore, it was possible to separate type 2 vWD patients from type 1 vWD patients on the basis of the shape and average diffusion time of the FCS curves. Cluster analysis yielded the expected separation of groups based on differences in the amount of antibody bound to antigen and the average diffusion time of bound antibody. Further analysis using a maximum entropy method FCS fitting program (MEMFCS) suggests further subclassification is possible with fluctuation analysis. The results indicate FCS is a practical tool for clinical evaluation of coagulopathic patients suspected of having von Willebrand disease. This research presents one of the first implementations of FCS in analysis of clinical samples.

3905-Pos

Dynamic Imaging and Fluctuation Spectroscopy on Single Microvilli in Opossum Kidney Cells by the Modulation Tracking Method

Luca Lanzano[1], Peter Fwu[1], Hector Giral[2], Moshe Levi[2], Enrico Gratton[1].
[1]Univ California, Irvine, CA, USA, [2]University of Colorado, Denver, CO, USA.

Regulation of renal tubular inorganic phosphate (Pi) transport occurs via the proximal tubular apical brush border membrane (BBM) sodium gradient-dependent Pi (NaPi) cotransport proteins. Distinct families of NaPi cotransporters show differential regulation under dietary and hormonal stimuli, but the way this is accomplished, for instance through localization in distinct BBM micro- or nano-domains and/or preferential interaction with different PDZ proteins, is not yet understood.

Crucial information could come from the application of single molecule fluctuation correlation spectroscopies on the BBM of living cultured Opossum Kidney (OK) cells expressing NaPi co-transporters with different GFP constructs. The BBM is composed of many microvilli, several micron long structures with a diameter of about 100nm. The microvilli show a relatively fast motion (in the seconds time scale) that makes the use of fluctuation spectroscopy difficult.

None of the current nano-resolution optical methods seems capable of measuring the clustering dynamics of proteins on the surface of rapidly moving microvilli. We developed an optical imaging technique called Modulation Tracking (MT) in which we track the center of mass of the microvillus at an arbitrary point along its length while the laser spot rapidly oscillates perpendicularly to the surface and the changes in the modulation are used to measure the distance of the spot from the fluorescent surface with nanometer resolution. High resolution images of the microvilli can be obtained scanning slowly along the microvillus axis. Since the moving microvillus is always at the center of the orbit, fluorescence image correlation techniques can be applied making the MT a truly dynamic nano-imaging technique.

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3906-Pos

Regulation of CFTR on the Plasma Membrane

Asmahan Abu-Arish, Ian R. Bates, Elvis Pandzic, Angela Ho,
John W. Hanrahan, Paul W. Wiseman.
McGill University, Montreal, QC, Canada.

The cystic fibrosis transmembrane conductance regulator (CFTR) is an anion channel which is tightly regulated by phosphorylation and interactions with a macromolecular complex that mediates spatially localized signaling mechanisms. The complex may include scaffolds such as NHERF1, the adaptors ezrin and Receptor for Activated C Kinase (RACK1), and enzymes such as adenylyl cyclase, kinases, phosphatases, and phosphodiesterases. NHERF1 anchors CFTR to the actin cytoskeleton whereas RACK1 mediates its association with protein kinase C (PKC), however the relationships between these proteins remain poorly understood. We have studied the dynamics of fluorescent fusion proteins containing CFTR, NHERF1 and RACK1 using quantitative fluorescence fluctuation imaging techniques. Lateral diffusion coefficients and immobility fractions at the plasma membrane were calculated from time-series of confocal images using temporal image correlation spectroscopy (TICS). We also developed a novel cross-correlation TICS (CC-TICS) analysis for studying the dynamics of interacting protein species and their binding ratios, so that the assembly and disassembly of the CFTR regulatory complex could be studied quantitatively. Initial results indicate that the lateral mobilities of RACK1 ($D=1.5\pm0.6\ \xi10^{-3}\ \mu m^2/s$) and NHERF1 ($D=2.6\pm1\ \xi10^{-4}\ \mu m^2/s$) are both significantly reduced ($D=8.6\pm2.5\ \xi10^{-4}$ and $1.8\pm0.8\ \xi10^{-4}\ \mu m^2/s$, respectively) when co-expressed with CFTR and are further reduced upon activation of PKC ($D=6.6\pm2.2\ \xi10^{-4}$ and $1\pm0.4\ \xi10^{-4}\ \mu m^2/s$, respectively). The fractions of immobility significantly increased whenever the diffusion coefficient decreased. The results suggest two distinct phases during CFTR complex formation; initial tethering under basal conditions followed by aggregation into complexes during PKC stimulation. These preliminary results provide new insight into protein-protein interactions that regulate CFTR, information that is essential for understanding anion transport in cystic fibrosis and secretory diarrhea.

3907-Pos

Predicting Protein Co-Expression Fractions in Living Cells

Elizabeth M. Smith, Yan Chen, Joachim Mueller.
University of Minnesota, Minneapolis, MN, USA.

Fluorescence fluctuation spectroscopy utilizes the fluctuation in a fluorescent signal to determine molecular brightness, concentration, and diffusion properties of fluorescent particles passing through an optical volume. Brightness analysis is

used to quantify the stoichiometry of protein association and to determine the number of proteins in a complex. In protein hetero-dimerization studies, it is highly desirable to be able to control protein co-expression fractions. The expression of each protein should be selected in order to maximize the likelihood of protein association. Yet protein expression is the output factor from a lipid-mediated transfection process in which only the plasmid DNA (input parameter) can be explicitly controlled. In this work we develop and test a working model which relates the protein expression fraction of two proteins to the plasmid DNA mixing fraction. Experimentally, we express two differently colored fluorescent proteins in CV-1 cells and split their emitted signal into two different detection channels based on color. The intensity fraction from the two channels is used to determine the relative amount of expressed protein in the cell. The experiments show that the DNA mixing fraction determines the average protein expression fraction. We extend this work by examining the cell-to-cell variation in protein expression and constructing a simple model which relates the expression variation to the average number of active plasmids. This study provides insight into the fundamentals of lipid-mediated transfection and demonstrates that we can control protein co-expression fractions through selection of plasmid DNA mixing fractions. This work is supported by NIH grant R01GM064589.

Molecular Mechanics & Force Spectroscopy II

3908-Pos

DNA Stretching Kinetics and Entropic Bottlenecks

Lorenzo Bongini[1], Pasquale bianco[2], Vincenzo Lombardi[2].
[1]University of Barcelona, Barcelona, Spain, [2]Università di Firenze, Sesto Fiorentino, Italy.

The use of a force clamp protocol during single molecule measurements of the DNA over-stretching transition by means of optical tweezers allow to experimentally access the kinetic of both DNA elongation and relaxation. The data are interpreted by means of a phenomenological two state model which allows to measure the cooperativity of the process. The comparison between experiments performed at different temperatures highlights the entropic nature of the free energy barrier that separates the compact and extended states of DNA. Insights on the structure of the intermediate state are provided.

3909-Pos

Multiple Binding Modes of Actinomycin D Reveal the Basis for its Potent HIV-1 and Cancer Activity

Thayaparan Paramanathan[1], Ioana D. Vladescu[2], Micah J. McCauley[1], Ioulia Rouzina[3], Mark C. Williams[1].
[1]Northeastern University, Boston, MA, USA, [2]Harvard University, Cambridge, MA, USA, [3]University of Minnesota, Minneapolis, MN, USA.

Actinomycin D (ActD) is a well studied anti-cancer agent that is used as a prototype for developing new generations of drugs. However, the biophysical basis of its activity is still unclear. Because ActD is known to intercalate double stranded DNA (dsDNA), it was assumed to block replication by stabilizing dsDNA in front of the replication fork. However, recent studies have shown that ActD binds with even higher affinity to imperfect duplexes and some sequences of single stranded DNA (ssDNA). These features suggest that ActD may alternatively destabilize complementary dsDNA. In this work we use optical tweezers to stretch and relax single dsDNA molecules in the presence of varying ActD concentrations. We observe that ActD binds with highest affinity to two separate DNA strands that are connected by ActD. This binding mode is ~1000-fold stronger than ActD's intercalation into dsDNA. We are able to characterize at least two classes of ActD-ssDNA binding sites that differ in dissociation times (~10% of sites with ~1000 sec off time, and the rest with ~10 sec off time). The much weaker ActD binding to dsDNA relative to ssDNA leads to duplex destabilization, in contrast to conventional intercalation. At saturation, the ActD-dsDNA complex becomes indistinguishable from the saturated ActD-ssDNA. These results suggest that two separate, anti-parallel DNA strands constitute the highest affinity natural substrate for ActD binding, with K_d ~10-100 nM and a relatively slow off rate. This finding supports the hypothesis that the primary characteristic of ActD that contributes to its biological activity is its ability to inhibit cellular replication by stabilizing DNA bubbles during RNA transcription, thereby stalling the transcription process.

3910-Pos

Atomistic Simulation of Estrogen Receptor-Coactivator Peptide Complexes to Identify Specific Binding Sites

Pradip K. Biswas[1], Rajendram Rajnarayanan[2].
[1]Tougaloo College, Tougaloo, MS, USA, [2]University at Buffalo, Buffalo, NY, USA.

Estrogen Receptors (ER) can lead to gene transcriptions that are found responsible for certain type of cancers including 70% of breast cancers. However, the process of gene transcription is preceded by the binding of an estrogenic ligand to the ER and then binding of this liganded ER with an activator protein which mediates the signal to the DNA. There are antiestrogenic compounds that can bind to the ER and block the binding of any coactivator protein to the liganded ER. However, no single antiestrogenic compound is found to work in all tissues - that means it cannot always block the binding of the coactivator protein. A successful drug molecule need to inhibit the liganded ER in all tissues. This needs a detail atomic level understanding of the interaction pattern of the liganded ER with coactivator protein. We investigate liganded ER interaction with small peptides to identify specific binding sites. Details of our investigation will be reported at the meeting. Acknowledgements: Authors acknowledge financial support from the National Institutes of Health (grant number 5P20MD002725-03-0002), through the NCMHD -RIMI program.

3911-Pos

Sources of Heterogeneity in the Forced Unfolding Pathway of Streptokinase Beta Revealed through High-Temperature Steered MD Simulations

K. Maria Mills[1], Dora Guzman[2], Zhibin Guan[2], Ioan Andricioaei[2].
[1]University of Michigan, Ann Arbor, MI, USA, [2]University of California Irvine, Irvine, CA, USA.

Force-pulling experiments on the unfolding of mechanical and non-mechanical protein domains have greatly increased our understanding of the structural stability of proteins. Because these experiments are done on the single molecule level, they also enable experimentalists to observe differences in the unfolding behavior of individual molecules. However, it is difficult to determine the source of unfolding heterogeneity through experiments alone. We present here evidence from experiments and simulations that the ß domain of Streptokinase, a non-mechanical protein, unfolds under force via three distinct pathways. High temperature SMD simulations were used to determine the source of the velocity-dependent heterogeneity observed in AFM force puling experiments. We show that hydrophobic interactions in the core of the protein underlie the differences observed in experiments and contribute significantly to the structural stability of the protein under force. Using an expansion of the Jarzynski equality[12], we calculate free energy surfaces to describe the energetics of the different pathways.

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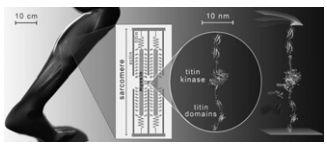
[2] D. Minh, J. Phys. Chem. B. **111**, 4137-4140 (2007)

3912-Pos

Exploring the Function of Titin Kinase by Mechanical Single-Molecule Pump-And-Probe

Elias M. Puchner, Hermann E. Gaub.
Ludwig-Maximilians-Universität München, Munich, Germany.

Protein function like catalytic activity or molecular recognition is tightly coupled to conformation and its dynamics. Since protein conformation can be controlled by forces, diverse mechanisms evolved allowing biological systems to respond to mechanical strain. Recently, it was shown in combination with single-molecule force spectroscopy, MD-simulations and enzymatics that titin kinase acts as a force sensor regulating muscle gene expression and protein turnover [1]. However, there is no experimental access to investigate which force-induced conformation during the activation pathway is competent for ATP-binding. Here we develop a new AFM-based single-molecule pump-and-probe protocol to mechanically prepare a predefined conformation and to read out afterwards whether ATP bound. We show that ATP only binds to the conformation prepared after barrier two. Therefore, titin kinase exhibits a dual mechanical autoinhibition, which cannot be overcome by thermal fluctuations but by physiological forces acting on the M-band structure of the muscle sarcomere. This single-molecule approach might also become useful for the investigation of other conformation controlled processes such as hidden binding pockets, catch bonds or motor proteins.



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3913-Pos

Single Molecule Study of the Motion of Matrix Metalloprotease MMP1 on Type I Collagen Fiber Shows Proteolysis Driven Hindered Biased Diffusion

Susanta K. Sarkar[1], Barry Marmer[2], Gregory Goldberg[2], Keir C. Neuman[1].
[1]Laboratory of Molecular Biophysics, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA, [2]Department of Biochemistry and Molecular Biophysics and Division of Dermatology, Washington University School of Medicine, St. Louis, MO, USA.

Diffusion plays an important role in many biological processes. Using single molecule fluorescence techniques, we have studied the diffusive motion of