was conducted. In generating starting conditions for the simulation special attention was paid to the backbone conformation since transitions between conformations were found to be rare events in a previous simulation of 100 ns length on this system [1]. Therefore, the experimentally determined conformation of the peptide backbone was equilibrated using a replica exchange technique, in an explicit membrane environment, to identify different confomers and their relative probability. Subsequently, the resulting distribution of conformations was used for a long conventional MD simulation that was analyzed with regard to the experimental data. Through such a combined approach a detailed model of the dynamics of the peptide was obtained.

References

[1]. Vogel, A.; Tan, K.-T.; Waldmann, H.; Feller, S. E.; Brown, M. F.; Huster, D. *Biophys. J.* **2007**, *93*, 2697–2712

2606-Plat PHLIP - pH (low) Insertion Peptide: Biophysical Studies and Medical Applications

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pHLIP (pH (Low) Insertion Peptide) is a membrane peptide with three states:

(I) soluble in a random configuration in water near neutral pH,
(II) bound in a random configuration to the surface of a membrane near neutral pH, and
(III) inserted across the membrane as an α-helix at acidic pH.

At low concentrations, pHLIP is a monomer in all three states. pHLIP insertion into lipid bilayers, human red blood cells and cancer cells *in vitro* and *in vivo* occurs as a result of protonation of Asp residues due to a decrease of pH. Protonation enhances peptide hydrophobicity and increases pHLIP affinity for a lipid bilayer 20 fold in comparison with neutral pH. The peptide does not induce fusion or membrane leakage. With low pH driving the process, pHLIP can translocate cargo molecules attached to its C-terminus via a disulfide and release them in the reducing environment of a cell cytoplasm. Among translocated molecules are fluorescent dyes, a hydrophilic cyclic peptide (phalloidin) and gene regulation agents (PNA, peptide nucleic acids). We have shown the ability of pHLIP to specifically target acidic tissue *in vivo* and to induce protein expression by PNA. The unique properties of pHLIP made it attractive for the biophysical investigation of membrane protein folding *in vitro* and for the development of a novel class of delivery peptides for the transport of therapeutic and diagnostic agents to acidic tissue sites associated with various pathological processes *in vivo*.

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2607-Plat Affinity of a Sphingolipid Binding Domain Peptide to Glycosphingolipid Receptors

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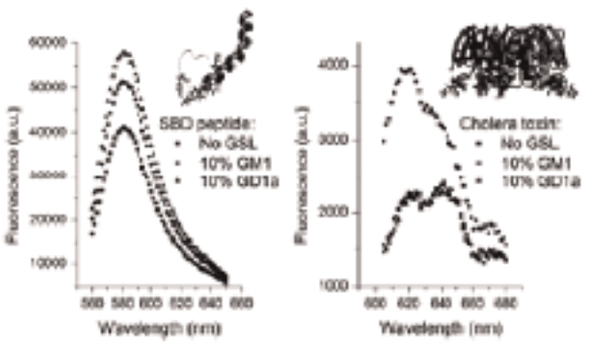
A fluorescently labeled peptide, the sphingolipid binding domain (SBD), is created as a tool to trace sphingolipid behavior and trafficking in neurodegenerative models. SBD, which consists of a 25 amino acid fragment of the Alzheimer's disease associated Aβ peptide, is TAMRA-coupled and applied to liposomes containing a raft-like mixture of sphingomyelin (SM), cholesterol (Chol), palmitoyl-oleoyl-phosphatidylcholine (POPC), and spiked with different glycosphingolipids. Such lipid compositions allow us to mimic a typical cell membrane with lipid microdomains. To assess the affinity of these different mixtures for SBD, unbound SBD are separated from liposomes by centrifugation. Fluorescence spectroscopy reveals a higher affinity of SBD for GD1a than for GM1; for comparison, fluorescently labeled cholera toxin binds as expected much more strongly to its receptor GM1 (Figure). A very poor affinity is found for control POPC-only liposomes, demonstrating the requirement of sphingolipid and cholesterol, the components of lipid microdomains, for binding. These peptide-bound microdomains will be investigated further by atomic force microscopy.

Figure. Fluorescence spectroscopy of liposomes with bound fluorescence-tagged SBD peptide (left) and cholera toxin (right). POPC/SM/Chol (45/25/30) liposomes were made with or without 10% in weight of glycosphingolipid receptors GM1 or GD1a.



Platform AY: Single Molecule Biophysics - III

2608-Plat Insights into the Nucleation of SH3 Amyloid Fibril Formation Using Single Molecule Fluorescence

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The tissue deposition of the [beta]-sheet rich, filamentous protein aggregates, amyloid fibrils, represents the common pathological hallmark of a range of degenerative disorders including Alzheimer's and Parkinson's diseases. However, the observation that many

proteins unrelated to disease can also form amyloid fibrils, suggests that the structural motif common to these aggregates is broadly accessible by polypeptide chains [1]. As such, developing an understanding of the mechanisms by which soluble protein molecules assemble into these fibrils is of fundamental and biomedical importance. Amyloid fibrils have been demonstrated to assemble typically via nucleation and growth kinetics, characterised by an initial lag phase prior to fibril elongation [2]. A wealth of data indicates that species formed during this phase of the reaction are cytotoxic, and furthermore, that soluble, oligomeric precursors to amyloid fibrils likely represent the critical pathological species in some amyloid disorders [3]. Probing the initial, stages of the assembly process is challenging due to the low populations of heterogeneous, unstable oligomeric species. To address this issue we have analysed the oligomers from an SH3 domain, molecule by molecule, using coincident bursts of fluorescence to distinguish oligomers from monomers. Our single-molecule results provide new insights into the stability of oligomer, the pathway to fibril formation and provide a general method to study the early events in the reaction.

References

[1]. Dobson, C. M. Nature *426*, 884–890 (2003).
[2]. Harper, J. D. & Lansbury, P. T. Annu. Rev. Biochem. *66*, 385–407 (1997).
[3]. Walsh, D. M. et al. Nature *416*, 535–539 (2002).

2609-Plat An Experimental Setup To Study The Activity Of Single Proteins In The Native State

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Studies of single protein turnover rate have lead to formulate an intriguing picture of the dynamics of proteins in their native state. Beginning with HRP and following with extensive studies on other proteins a scenario has been built up in which the native state of a protein is formed by an enormous amount of very similar configurations with slightly different catalytic activity. A protein is continuously hopping from one state to the other and the commonly observed Michaelis-Menten behaviour is the result of averaging both dynamic (average over time) and static (average over protein population) disorder.

Little is known on the number of these near-native configurations, and little is know on how this number varies with external parameters such as temperature or external force.

We have built an experimental setup aimed at addressing precisely those questions. I will describe in details the development of this experimental setup and I will present some preliminary results on the measurement of single protein activity.

2610-Plat Single-Molecule Methods for Examining Protein Interactions on the Surface of Living Cells

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Developing physical methods to examine protein interactions in living cells presents a considerable challenge. We have recently developed a new tool applicable to such studies, single-molecule fluorescence two-colour coincidence detection spectroscopy (TCCD), a technique initially applied to molecules in free solution [1]. The methodology is based upon detecting individual fluorescent bursts from red or blue fluorophore-tagged molecules, as they diffuse through overlapped red and blue confocal laser volumes. Coincident fluorescent events derive from associated molecules, and from non-associated molecules randomly occupying the probe volumes simultaneously. Analysis of the data identifies bursts above an optimised threshold, and simple statistical considerations allow the contribution from chance coincidence to be subtracted [2]. We have validated our method using known monomeric and dimeric proteins on the surface of a live T cell, and then applied the method to probe interactions in the membranes of living cells, between protein pairs whose association-state is unclear [3].

References

[1]. Li, H.T. *et al.*, *Analytical Chemistry*, **2003**. *75*(7): p. 1664–1670
[2]. Clarke, R.W. *et al.*, *Analytical Chemistry*, **2007**. *79*(7): p. 2771–2777
[3]. James, J.R. *et al.*, *PNAS* (*in press*), **2007**.

2611-Plat Dynamic Regulation Of PI (3,4,5)P_3 Phosphatase Activity Of PTEN On Membranes Revealed By Single-molecule Imaging

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Chemotaxis of eukaryotic cells involves an establishment of cellular polarity along the moving direction, and one hallmark is a localization of PI(3,4,5)P_3 on membranes at the leading edge. By acting at lateral and posterior membranes, the PI 3-phosphatase, PTEN, plays an indispensable role in restricting PI(3,4,5)P_3 to the front. We have revealed the mechanisms of regulation of PTEN by observing single molecules of Halo-tagged PTEN in living *Dictyostelium* cells. Single PTEN molecules showed transient association and lateral diffusion on membranes. From an analysis of the lifetimes of membrane interaction, we revealed two populations of membrane-bound PTEN. These two populations distributed on membranes in a spatially regulated manner along cell polarity. The population with the slower dissociation rate was detected exclusively at posterior membrane, while the faster one was observed at whole membrane. From an analysis of the lateral diffusion, we

found membrane-bound PTEN undergoes a transition between two states with different diffusion coefficients. The difference in the dissociation rate could be attributed to the difference in the transition rate to the state with the slower diffusion. The transition likely involves the interaction with some membrane-associated molecule. It is possible that this unidentified molecule enriched on posterior membrane controls the membrane-binding stability of PTEN molecules on membrane, which is a novel mechanism how PTEN is locally activated on the posterior membrane.

2612-Plat Probing the ClpX ATPase with Optical Tweezers and Single Molecule Fluorescence

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Protein integrity inside cells is maintained by proteolytic machines capable of recognizing and engaging substrates targeted for destruction. In E. coli, one such protease, ClpXP, is composed of the ClpX ATPase and the ClpP peptidase, which are responsible for unfolding and denaturing substrates, respectively. Even though ClpX has been the focus of much experimentation at the ensemble level, fundamental questions about its mechanisms of action remain unanswered. Here we present a novel immobilization strategy used to develop the first single molecule assays for this ATPase. In the first assay, which employs single molecule fluorescence, ClpX was found to unfold and translocate pre-engaged GFP substrates with a time constant of 22 s at saturating ATP concentrations. This rate is 8-fold faster than bulk measurements, which can be clouded by binding and unbinding events. The second assay measured the strength of the ClpX-substrate interaction with optical tweezers. It revealed that ClpX holds on to its substrates with forces on the order of 60 pN regardless of the nature and concentration of the nucleotide in solution. These assays can be easily modified to further test the mechanistic behavior of ClpX and a host of other enzymes to gain a better understanding of proteolytic machinery.

2613-Plat Subunit Organization Of The Voltage-gated Proton Channel Hv1 And The Voltage-gated Phosphatase Ci-VSP

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The newly discovered voltage-gated proton channel Hv1 contains a voltage sensing domain with transmembrane segments S1–S4 like voltage-gated K+ channels, but is lacking the pore domain with S5–S6 which is supposed to mediate tetramerization in K+ channels. Similarly, in the recently found voltage-dependent phosphatase Ci-VSP the pore domain is replaced by a cytosolic enzymatic domain. The oligomerization states of Hv1 and Ci-VSP are unknown. In order to determine the number of subunits of these proteins, we used a method we developed recently, where a fusion of GFP and the target protein is expressed in Xenopus laevis oocytes and photobleaching steps of GFP are observed on a single molecule level. Simultaneous imaging of many single fluorescent protein molecules results in a distribution of photobleaching steps that allows for the determination of the oligomerization state of the protein without the need of protein overexpression. We discovered that Ci-VSP is monomeric and that Hv1 is multimeric. We will present experiments that determine the subunit number of the Hv1 multimer and that look for the molecular determinants that mediate Hv1 multimerization by generating chimeric proteins between Hv1 and Ci-VSP.

2614-Plat Combining Single Molecule Fluorescence and Microfluidics to Explore Protein Folding and Binding

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Protein folding, a reaction involving complex energy landscapes and dynamics on various timescales, is often difficult to probe in detail using standard ensemble experiments. In contrast, single-molecule FRET (smFRET) allows monitoring of distributions of protein folding states one molecule at a time. However, most smFRET folding measurements of freely diffusing proteins are currently restricted to observations at equilibrium conditions. Kinetic studies of protein folding are typically carried out at ensemble level, using stopped-flow devices, which use turbulence to rapidly alter denaturant concentration. A more recent approach has been to use microfluidic mixing in a laminar flow, which provides reduced dead-time and minimal sample consumption. Such methods are still mostly limited to ensemble measurements. Protein folding kinetics combined with the power of single-molecule analyses can potentially reveal more detailed information about novel folding landscape features such as the presence of intermediates and multiple folding pathways.

We have developed microfluidic devices that include an ultrafast mixer for microsecond mixing and ensemble FRET studies of folding kinetics, immediately followed by a controlled flow that allows single molecule fluorescence detection. Made of a single cast of PDMS, the chips are inexpensive to fabricate and easy to operate. Our device provides high time-resolution records of non-equilibrium folding distributions and dynamics at single molecule resolution, as demonstrated by studying the folding of alpha-synuclein induced by its binding to liposomes.

2615-Plat Diffusion Constant of a DNA Sliding Clamp Measured by Single Molecule Fluorescence Spectroscopy

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DNA sliding clamps attach to polymerases and slide along DNA to allow rapid, processive replication of DNA. These clamps contain

many positively charged residues which could curtail the sliding due to attractive interactions with the negatively charged DNA. By single-molecule spectroscopy we have observed a fluorescently-labeled sliding clamp (pol III β subunit or β clamp), loaded onto freely diffusing, single-stranded M13 circular DNA annealed with fluorescently labeled DNA oligomers of up to 90 bases. We use solution-based single molecule fluorescence resonance energy transfer (FRET) experiments. We analyze the results using burst analysis and purified fluorescence correlation spectroscopy (PFCS). We find that the diffusion constant for the β clamp diffusing along DNA is on the order of 10^{-14} m^2/s_at least three orders of magnitude less than that for diffusion through water alone. We also find that the β clamp remains at the 3′ end in the presence of *E. coli* single-stranded binding protein (SSB). These results may imply that the clamp not only acts as a tether, but also a placeholder.

Platform AZ: Anion Channels

2616-Plat The ClC-0 Chloride Channel Is a "Broken" Cl^-/H^+ Antiporter

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ClC-0 is the quintessential CLC chloride channel. Previously, ClC-0 gating was shown to be a non-equilibrium process [1]. It was proposed that the source of energy driving this process is the chloride electrochemical gradient. However, although this explanation was qualitatively satisfying, it failed to quantitatively account for the data. Since there appeared to be no other source of energy in the system, this created a mystery. Inspired by the recent discovery that some of the CLC proteins are Cl^-/H^+ antiporters [2, 3, 4] we revisited this problem by testing the hypothesis that proton movement powers the non-equilibrium gating in ClC-0. Using single-channel recordings, we show that the proton electrochemical gradient quantitatively accounts for the observed non-equilibrium gating. These results indicate that ClC-0 gating catalyzes proton transport and suggest that ClC-0 is a "broken" Cl^-/H^+ antiporter [5], in which one of the conformational states has become leaky for chloride ions. Currently, we are conducting experiments to examine specifically the conformational changes associated with the gating.

References

[1]. Richard, E. A., and Miller, C. (1990) *Science 247*:1208.
[2]. Accardi, A., and Miller, C. (2004) *Nature 427*:803.
[3]. Picollo, A., and Pusch, M. (2005) *Nature 436*:420.
[4]. Scheel, O., *et al.* (2005) *Nature 436*:424.
[5]. Miller, C. (2006) *Nature 440*:484.

2617-Plat R-helix Couples Movements in the C-terminal Domain to the Pore of CLC-0

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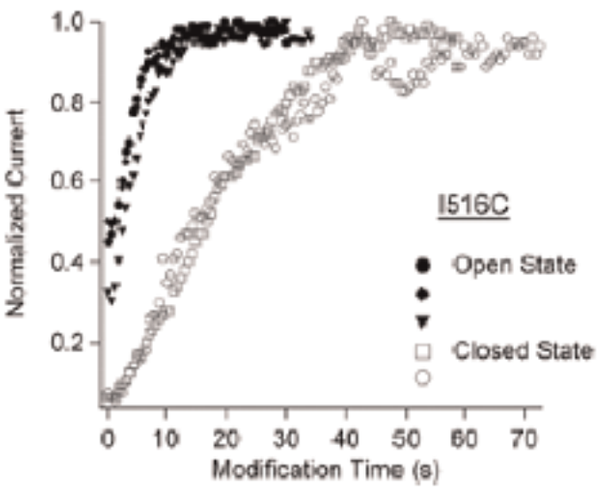
The common (slow) gate of CLC-0 generates long silent periods in single-channel recordings. Our previous FRET measurements suggest that common gating involves a large movement of the C-terminal domain. How do movements in the intracellular region affect chloride permeation through the membrane-spanning core? To try to answer this question, our current study focuses on R-helix, a pore-forming structure that directly links the C-terminal domain to the chloride permeation pathway. When the common gate is locked in its open state (with C212S), cysteine mutations in R-helix have little effects on fast gating. However, these same mutations strongly affect the kinetics as well as equilibrium of common gating in the wildtype channel background. Furthermore, the modification rate of R-helix cysteines is strongly dependent on the state of the common gate. These results indicate that R-helix has an important role in common gating.



2618-Plat Thermodynamics Of Anion Binding To a ClC Transporter

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CLC-ec1 is a Cl^-/H^+ antiporter of the CLC family. In a transporter substrate binding triggers a cascade of conformational changes whose result is ion translocation across the membrane. Two parameters characterize a transport system: its transport rate and the affinity of substrate. Little is known about the thermodynamic properties of substrate binding to CLC-ec1. The crystal structures show that Cl^- can bind to three sites that define a pathway through the protein. In the WT the inner and central sites are occupied by Cl^- while the external one is occupied by E148. We used Isothermal Titration Calorimetry to measure Cl^- binding to CLC-ec1 and found it has a K_d of ~0.5 mM, ~6 times lower than previously reported. Additionally, we found that Br^- and NO_3^- have K_d's ~2.2 mM and ~5 mM respectively. This selectivity preference closely mirrors the permeability sequence estimated by electrophysiological measurements. To determine the Cl^- affinities of the individual sites we used mutants that destabilize one or more binding sites like E148A, Y445A and E148A/Y445A. We found that Cl^- binds to the E148A