integral membrane proteins known as urea transporters (UTs). While the structure of a bacterial homolog was solved previously, the bacterial UT transports urea much slower than the mammalian ones and its similarity to the mammalian proteins remained unclear. In addition, little was known of the mechanism of permeation. To answer these questions, we have solved the first structure of a mammalian urea transporter at 2.4 Å, and probed the energetics of urea permeation with a combination of molecular dynamics simulations, functional characterization of mutants and co-crystallization with urea analogs. Similarities with the bacterial UT suggest that many features of the structure are broadly conserved across the UT family, including a trimeric assembly, and the presence of a channel-like, continuous permeation pathway through each monomer. The functional and computational studies revealed a large energy barrier at the center of the permeation pathway, whose presence may hint towards a possible gating mechanism.

Platform: Fluorescence Spectroscopy

1090-Plat

Unraveling Folding Pathways and Kinetics Transition of T4 Lysozyme with High Temporal Resolution by Single Molecule FRET

Hugo Sanabria[1], Dmytro Rodnin[1], Suren Felekyan[1], Stanislav Kalinin[1], Mark Fleissner[2], Wayne Hubbell[2], Claus A.M. Seidel[1].
[1]Heirich Heine Universität, Düsseldorf, Germany, [2]University of California, Los Angeles, CA, USA.

Protein folding is directly connected to protein function, and miss folding events could lead to disease. Currently, one of the challenges of understanding protein folding is to determine the number of meta stable or excited intermediate states and their corresponding kinetic pathways during folding transitions given the large number of possible accessible conformers. Therefore, we combine the power of single molecule FRET, with site specific labeling, filtered fluorescence correlation spectroscopy (fFCS), multiparameter fluorescence detection (MFD) and ensemble time correlated single photon counting to determine the folding and unfolding kinetics and the corresponding reaction pathways of T4 Lysozyme (T4L) under various chemical denaturation conditions (Urea, GdmCl, and pH) with sub microsecond resolution.

MFD, a single molecule technique, allows the direct observation of populations; even when low populated (<10%). fFCS, a recently developed modification of the standard FCS, uses the fluorescence lifetime and anisotropy decays to filter the signal and properly weight the contribution of each photon to the corresponding population in an heterogeneous solution. The weighted time dependent signal with specific spectral window and polarization is correlated to obtain all possible auto-correlation and cross-correlation curves. Of great utility is the cross-correlation curve because it resolves, with maximum contrast, the anticorrelated behavior of the interconversion between populations.

Using the afore mentioned toolbox, we observe that each denaturant results in different folding and unfolding pathways in T4L. Furthermore, the addition of surfactant (Tween 20) changes dramatically the reaction coordinate in the folding and unfolding energy landscape even in the same denaturant conditions. One of the major differences observed is that pH denaturation showed the accumulation of an intermediate state where most likely the N terminal domain is partially unfolded while the C terminal domain remains folded.

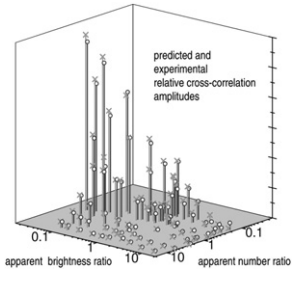
1091-Plat

Correcting for Artifacts from Spectral Cross-Talk and Imperfect Detection Volume Overlap in Dual-Color Fluorescence Cross-Correlation Spectroscopy

Kirsten Bacia[1], Stefan Werner[1], Zdenek Petrasek[2], Petra Schwille[2].
[1]Halomem, University of Halle, Halle, Germany, [2]Institute of Biophysics, BIOTEC, TU Dresden, Dresden, Germany.

Dual-color fluorescence cross-correlation spectroscopy (dcFCCS) allows to quantitatively assess the interactions of mobile molecules labeled with distinct fluorophores.

One major artifact risk in dcFCCS is a false-positive or overestimated cross-correlation amplitude arising from spectral cross-talk. Cross-talk can be reduced or prevented by fast alternating excitation, but the technology is not easily implemented in standard commercial setups.



We devised an experimental strategy that does not require specialized hard- and software for recognizing and correcting for cross-talk in standard dcFCCS. Another major artifact risk in dcFCCS is a false-negative or underestimated cross-correlation amplitude arising from an imperfect detection volume overlap. Samples based on fluorophores conjugated to oligonucleotides that have been traditionally used for dcFCCS calibration typically suffer from incomplete labeling. Using these samples, it is difficult to attribute a reduced cross-correlation amplitude to the imperfection of the calibration sample or to that of the setup. We therefore developed a new cross-correlation calibration standard with a predictable degree of labeling.

1092-Plat

Confined Dynamics and Membrane Domain Features Revealed by Image Correlation Spectroscopy Toolkit

Elvis Pandzic, Paul W. Wiseman.
McGill University, Montreal, QC, Canada.

The modern model of the cell membrane posits that it is spatially heterogeneous with domains that restrict molecular transport. Lipid rafts, or microdomains, are one such membrane domain that are enriched in sphingolipids, and function to sequester proteins in transient complexes that are believed to be a few 10s to 100s of nanometers in size. Membrane proteins may also be sequestered by the membrane proximal actin cytoskeleton. Here we show that image correlation based techniques, applied to standard laser scanning or TIRF fluorescence microscopy image series, can be used to discriminate between the different mechanisms of confined diffusion. We plot the diffusion time lag versus the waist of the correlation functions at a given time lag, to obtain the Mean Square Displacement (MSD) from the correlation function width, which is analogous to the previously described FCS diffusion law for variable beam radii. The ICS approach has the advantage of being based on image analysis without the need to vary the beam size. Furthermore, we show how one can extract from the correlation function data the characteristic parameters of the system such as domain size, density, diffusion coefficients and partition rates. To verify the validity of this tool, we performed simulations of confined diffusion in meshwork and microdomains where we varied the domain size (10–1000 nm radius), density (up to 10 % area coverage), confinement probability and diffusion coefficients (0.002–0.1 μm2/s). We used the simulations to establish the limits due to the spatio-temporal sampling and noise. We applied this analysis to the study of dynamics of membrane proteins known to be raft associated: GPI-GFP and Cholera Toxin Subunit B, and verified that our tools can detect changes in the confinement parameters following the application of drugs that disrupt rafts.

1093-Plat

Tracking of Single Microvilli to Study Regulation of the Intestinal Phosphate Transporters

Luca Lanzano[1], Yupanqui Caldas[2], Hector Giral[2], Michelle Digman[1], Moshe Levi[2], Enrico Gratton[1].
[1]University of California, Irvine, CA, USA, [2]University of Colorado Denver, Aurora, CO, USA.

Intestinal phosphate (Pi) uptake is one of the key mechanisms of systemic Pi homeostasis. Modulation of intestinal Pi transport has been recently recognized as an important target in prevention of hyperphosphatemia and the associated cardiovascular complications in Chronic Kidney Disease. Intestinal Pi absorption is mainly mediated by the type IIb sodium-phosphate co-transporter NaPi2b which is expressed in the intestinal apical microvilli. Recent evidences show that agonists of the LXR nuclear hormone receptor inhibit intestinal NaPi transport activity, NaPi2b protein and mRNA abundance, and decrease serum Pi level [1]. However, the detailed mechanisms of how LXR decreases intestinal NaPi transport activity, including the role of changes in membrane cholesterol or PDZ interacting proteins, remain to be determined.

In order to study these processes at the molecular level we apply the Modulation Tracking nanoimaging method [2] to microvilli of live cells expressing fluorescently tagged NaPi2b. This technique generalizes the principles of Single Particle Tracking to allow the imaging of extended subcellular structures. Tracking the single microvilli we directly measure their motion, while we also image NaPi2b distribution along the membrane and perform Fluorescence Correlation Spectroscopy techniques (RICS, N&B) in steady state, even if the microvilli are moving. Different dynamic properties, including motility of the microvilli and protein diffusion and clustering, are monitored under stimulation to get insights into the process of intestinal transporters regulation.

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