high-quality data at the HTS level is vital to the success of the subsequent steps, which are much more expensive and lower in throughput. The HTS data must be of sufficiently high quality, with low incidence of false positives and negatives, in order to establish an effective roadmap for the subsequent processes. Too often, the emphasis is on rapidly screening huge libraries (''quick and dirty''), producing inefficiencies in subsequent steps. By taking advantage of new fluorescence lifetime measurement technology, powerful new assays are enabled. Our premise is that time-resolved FRET (TR-FRET) is so highly advantageous over intensity-based assays that it should always be the first choice. TR-FRET on the nanosecond time scale is not the norm now. The most familiar form is live cell assays with genetically encoded fluorescent proteins, which are generally approached by FLIM. However, the low speed of FLIM makes it applicability for HTS very limited. Our solution is to employ a ''Cells-in-Wells'' strategy in which the collective response of hundreds of cells are monitored simultaneously in an non-imaging format. We will present data that demonstrates the conversion of assays that are useless for HTS in an intensity mode ($z' < 0$) to very robust assays ($z' > 0.7$) in fluorescence lifetime mode.

2419-Pos Board B189

Effects of Macromolecular Crowding on Protein Biophysics

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Proteins fold and function inside cells which are environments very different from that of a dilute buffer solution most often used in *in vitro* experiments. The cell compartments are full of other proteins, membranes and DNA; it is estimated that up to 40 % of the available volume in a cell can be occupied by other biomolecules. The crowded environment results in increased viscosity, excluded-volume effects and amplified opportunity for specific and non-specific inter-molecular interactions. These environmental factors are not accounted for in the mechanistic studies of protein folding and function that have been executed during the last decades. The question thus arises as for how these effects - present when polypeptides normally fold *in vivo* - modulate protein biophysics? To take a step closer to understanding the *in vivo* scenario, we here assess how crowded environments affect protein biophysical properties. For this we use synthetic macromolecular crowding agents, which take up significant volume but do not interact with the target proteins, in combination with strategically selected proteins and a range of biophysical/spectroscopic methods. We have found that in the presence of macromolecular crowding *in vitro*, proteins become more thermodynamically stable (magnitude depends inversely on protein stability in buffer) and, protein folded states may change both secondary structure content and overall shape. For a protein with a complex folding mechanism involving an off-path intermediate, excluded volume effects make the folding energy landscape smoother (*i.e.,* less misfolding) than in buffer. Our findings demonstrate that excluded volume effects tune protein biophysical parameters: this is of mechanistic relevance since proteins have evolved to fold and function in crowded environments.

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Localization of the Escherichia Coli Divisome by Nucleoid Occlusion and Membrane Curvature

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Cell division in typical rod-shaped bacteria such as Escherichia coli shows a remarkable plasticity in being able to adapt to a variety of irregular cell shapes. Here, we investigate the roles of the Min system and nucleoid occlusion mechanism in supporting this adaption. We study 'squeezed' E. coli in shallow nanofabricated channels [1], which despite their highly irregular shapes and large areas, are able to divide into two almost equally sized daughters and we compare this phenotype to normal rod-shaped cells, and mutants carrying deletions in Min and nucleoid occlusion systems. Fluorescently labeled MinD proteins in aberrant cell shapes show an irregular pattern of movement and do generally not generate a reliable signal for localization of the cell division proteins. By contrast, we establish that nucleoid occlusion provides a robust molecular mechanism which is not sensitive to perturbations in cell shapes. We also find that membrane curvature is important in selecting the nucleation site for the divisome. Progressive FtsZ arcs form only in those positions on the circumference of the squeezed E. coli where their line curvature is maximized. Our study underscores the importance of two so far poorly understood mechanisms - nucleoid occlusion and membrane curvature, which E. coli uses to localize their cell division apparatus in multiple of phenotypes.

[1] J. Männik, R. Driessen, P. Galajda, J.E. Keymer and C. Dekker, Proc. Natl. Acad. Sci. USA. 106 (2009) 14861.

2421-Pos Board B191

Magnetic Manipulation of Signaling "Hotspots" Inside Living Cells Shows Context-Dependent Amplification of the Rac Pathway

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In vivo imaging has shown how the establishment and maintenance of a polarized cellular state relies on complex mechanisms by which signaling cascades become activated and regulated at sub-cellular levels. It has recently led to the concept of spatially-restricted signaling module. Yet, it remains unclear what combination of molecular players, dynamics and function specify a module. How they become integrated and spatio-temporally coordinated into a polarized cell is also not elucidated. In this context, we used a new tool to locally perturb and probe the signaling pathways associated to small Rho-GTPases.

First, magnetic nanoparticles (500nm), functionalized with constitutively active Rho-GTPases (Rac1 and cdc42) were microinjected inside living cells. We observed the induction of downstream signaling at the particle surface by visualization of effectors binding (Pak and N-WASP) and the local polymerization of actin structures, thereby demonstrating that these particles behave in the cytosol as signaling ''hotspots''. Using FRAP, we further measured in vivo dissociation rates between the immobilized GTPase and its direct effector. Next, the nanoparticles were manipulated with a magnetic tip to position their signaling activity at different subcellular locations. With nanoparticles functionalized in situ with Tiam (a known Rac1 activator), we were able to specifically activates Rac1 signaling by bringing the nanoparticles in contact with the plasma membrane. The Rac1 activation occurred regardless of the subcellular localization at the membrane. However, the signal was non-linearly amplified only when the signaling activity was carried to protrusive areas of the cell. This result reinforces the concept of subcellular signaling modules and shows that a module could be specified by its ability to amplify rather than just linearly transduce incoming signals.

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From Single-Molecule Interactions to Population-Level Dynamics: Understanding the Complex Organization of RNA Pol II in the Nucleus of Living Cells

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Transcription involves a complex exchange within a reservoir of proteins in the nucleoplasm, and the specific recruitment of individual proteins at specific gene loci. However, understanding the spatial distribution of individual proteins and the temporal behavior in the nucleus of living cells remains challenging. Using 3D super-resolution fluorescence microscopy and cluster analysis, we observe that the distribution of RNA Polymerase II (Pol II) cluster sizes, measured as the number of polymerases per cluster, follows a $-3/2$ power law. Radial dependent analysis of the spatial distribution of Pol II also shows scale-invariance, consistent with a so-called self-organized criticality in a fractal geometry of dimension ~2.7. These results suggest a diffusion-based mechanism whereby, via transient interactions, massive recruitment and dismissal of pol II molecules can occur at specific loci in the nucleoplasm. Kinetic measurements using single-molecule detection in live cells reveal Pol II binding dynamics within minutes. Serum-induced transcription increased Pol II binding kinetics in live cells by an order of magnitude. Together, these results provide a comprehensive view of the spatio-temporal organization of Pol II in the nucleus: from the global population distribution, to single molecule recruitment at specific loci in live cells. This comprehensive single-cell approach can be adopted for other proteins beside RNA Pol II, for real-time quantification of protein organization in vivo, with single-molecule sensitivity.

2423-Pos Board B193

Enzyme Selective Flim in Microglia: Studying the Role of NADPH Oxidase in Chronic Neuroinflammation

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Understanding the pathological mechanisms in the brain is the decisive step in developing novel specific therapeutic strategies and in curing chronic neuroinflammatory or neurodegenerative diseases like multiple sclerosis (MS).