two unfolding transitions. The refolding process also shows one or two refold-
ing steps, suggesting the existence of a stable intermediate unfolding/refolding state. The total unfolding distance is about 5.4 nm, consistent with the expected unfolded G4 structure. However, when the DNA molecules were pre-incubated with a G4 stabilizing ligand BVMC (3,6-bis(1-methyl-4-vinylpyridinium) carbazole), most DNA molecules showed three unfolding and refolding transitions, suggesting of two unfolding/refolding intermediate states. Our observation suggests that BVMC-bound G4 structures unfold and refold in two unfolding/refolding intermediates, in contrast of the one in the ligand-free G4 structure.

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Controlled twisting of individual, double-stranded DNA molecules provides a unique method to investigate the enzymes that alter DNA topology. Such twisting requires a single DNA molecule to be torsionally constrained. This constraint is achieved by anchoring the opposite ends of the DNA to two separate surfaces via multiple bonds. The traditional protocol for making such DNA involves a three-way ligation followed by gel purification, a laborious process that often leads to low yield both in the amount of DNA and the fraction of molecules that are torsionally constrained. We developed a simple, ligation-free procedure for making torsionally constrained DNA via polymerase chain reaction (PCR). This PCR protocol used two “megaprimer”, 400-base-pair long primers to generate double-stranded DNA that were labelled with either biotin or digoxigenin. We obtained a relatively high yield of gel-purified DNA (~500 ng per 100 ng PCR product). The final construct in this PCR-based method contains only one labelled strand in contrast to the traditional construct in which both strands of the DNA are labelled. Nonetheless, we achieved a high yield (84%) of torsionally constrained DNA when measured using an optical-trap-based DNA-overstretching assay. This protocol significantly simplifies the application and adoption of torsionally constrained assays to a wide range of single-molecule systems.

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Single-Molecule Analysis of Hepatitis C Virus NS3 Helicase Translocating on Single-Stranded RNA

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Large, structured RNA molecules often require the need of RNA helicases in order to correctly and efficiently fold into their active conformation. Likewise, RNA helicases are needed to displace proteins from RNA molecules or to melt RNA structures to make parts of the RNA accessible for binding partners. Long-range translocation events of RNA helicases on their substrates are functionally important, yet lack understanding at the single molecule level. Using the RNA helicase NS3h from Hepatitis C Virus (DeH/D-box family) we used a custom-built “feeder”, a single-molecule instrument combining fluorescence detection with an optical tweezer, to observe, identify and define underlying translocation events. This instrument provides the necessary means to locate a fluorescence-labeled molecule with the precision of a few nanometers on a long nucleic acid strand that is held under a defined and adjustable tension with piconewton accuracy by optical trapping. We measured long-range translocation of HCV NS3h on single-stranded RNA of various sequences and obtained data on velocity and processivity. With ca. 70 nucleotides per second, NS3h translocation on single stranded RNA is 2-2.5 times faster than on single stranded DNA.

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Counting Small RNA in Pathogenic Bacteria

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We present a modification to single-molecule fluorescence in-situ hybridization (smFISH) that enables quantitative detection and analysis of small RNA (sRNA) expressed in bacteria. We show that short (~200 nucleotide) nucleic acid targets can be detected when the background of unbound singly-dye labeled DNA oligomers is reduced through hybridization with a set of complementary DNA oligomers labeled with a fluorescence quencher. By neutralizing the fluorescence from unbound probes, we were able to significantly reduce the number of false positives, allowing for accurate quantification of sRNA levels. Exploiting an automated, multi-color wide-field microscope and data analysis package, we analyzed the statistics of sRNA expression in thousands of individual bacteria. We found that only a small fraction of either Yersinia pseudotuberculosis or Yersinia pestis bacteria express the small RNAs YSR35 or YSP9, with the copy number typically between 0-10 transcripts. The numbers of these short RNA are both increased (by a factor of 2.5x for YSR35 and 3.5x for YSP9) upon a temperature shift from 25°C to 37°C, suggesting they play a role in pathogenesis. The copy number distribution of sRNAs from bacteria-to-bacteria are well-fit with a falling model of gene transcription. The ability to directly quantify expression levels changes of sRNA in single cells as a function of external stimuli provides key information on the role of sRNA in cellular regulatory networks.

Optical Microscopy and Super Resolution Imaging II

2001-Pos Board B731

Insight into Hybrid Nanoscopy Techniques: STED AFM & STORM AFM

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Microscopy as we know today has improved its resolution below the classical diffraction limit by introducing a field named ‘nanoscopy’ or super-resolution microscopy like STED, STORM, SIM etc. Correlative techniques pose as a new trend in microscopy for their ability to confirm a dataset from one method compared against another, hence forming concordant information. With this idea in mind of a multi-capable system, we coupled three biomedical tools into one hybrid microscope, which can fire a set of questions to a sample of interest in a single go. Last year, our group demonstrated this coupling of STED AFM FCS as a feasible correlative technique which can pose multidimensional questions to a sample in a [gol][1][2]. Here, we present a new hybrid tool: STORM AFM and its comparative results with STED AFM [3]. We also demonstrate how this concept of correlative microscopy can be taken ahead for spectroscopic investigations using FCS and how these information contents are bewildering to the scientific perceptions of the nano-world, specifically for the study of lipids, model membranes and cell nucleus structure. Our focus is in asserting that the key elemental differences in the super resolution hybrid approaches can be useful in facing problems related to diverse biological environments like membrane architecture or cellular dynamics by suiting the correlative probes.

References:

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Correlating Mobility and Interaction of Transcription Factors by SPIM-FCS

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In single plane illumination microscopy (SPIM) an image of a two-dimensional slice of a sample is formed by illumination with a laser beam focused in one dimension. Thus, fluorescence fluctuations may be observed simultaneously in all voxels of this 2D slice, thereby obtaining diffusion and transport coefficients as well as concentrations of fluorescently labeled biomolecules, creating a “mobility map” of the observed cell. By combining SPIM fluorescence correlation and cross-correlation spectroscopy (FCS/FCCS), direct information on the strength of molecular interactions can be obtained. We built a dual color SPIM-FCCS with two laser wavelengths for illumination and dual-channel detection through either an EMCCD camera or an ultrafast avalanche photodiode matrix detector, with wavelength splitting optics to image the two colour channels simultaneously. This system offers a time resolution in the range of 106 frames/sec.
Here we demonstrate that SPIM-FCS/FCCS possesses the sensitivity to detect and quantify protein-protein interactions in live cells by characterizing the interaction of the subunits of heterodimeric transcription factors, c-Fos/c-Jun and IQGAP/cdc42. The protein-protein interaction clearly shows up in the cross-correlation amplitude. Analysis of the spatial distribution of difffusion coefficients of the fluorescent proteins and of their cross-correlation amplitude shows that formation of the heterodimer is correlated with regions of decreased mobility, probably related to DNA binding.

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Super-Resolution Fluorescence Imaging Reveals Nanoscale Organization of Stress Granule
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In response to environmental stresses, cytoplasmic mRNAs assemble and form stress granules (SGs) accompanied by several proteins affecting mRNA functions. SGs have been proposed to play an important role in mRNA remodeling for repression of translation. However, the underlying mechanism remains unclear, since conventional fluorescence imaging cannot resolve the detailed distributions of SGs components due to the diffraction limit. Here, we investigated nanoscale organization of SGs by stochastic optical reconstruction microscopy (STORM), which provided super-resolution images with spatial resolution of ~20 nanometers in the lateral direction and of ~60 nanometers in the axial direction. Super-resolution imaging revealed that mRNAs in SGs were highly localized to tiny compartments with a diameter of less than 100 nanometers, whereas they were elusive in conventional fluorescence imaging. In mature SGs, the number of these compartments was higher than in small SGs, but the size of these compartments showed little difference. The result demonstrated that the growing process of SGs resulted from the assembly of tiny compartments. Furthermore, multicolor super-resolution imaging showed that some SG associated proteins colocalized with mRNAs and others did not. Since SGs components were densely packed within several micrometers radius, we could not observe the differences by conventional fluorescence imaging. Thus, we could not observe the differences by conventional fluorescence imaging.

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Glycan Catabolism by Human Gut Symbionts involves Dynamic Protein Interactions
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The human gut hosts trillions of bacteria that directly influence human health. The majority of gut microbiota play an important role in nutrition by metabolizing undigestible complex carbohydrates into short-chain fatty acids. Bacteroides thetaiotaomicron (Bt), a prominent bacterial symbiont in the distal gut, metabolizes over a dozen complex glycans using membrane-associated protein complexes. The Starch Utilization System (Sus), a multi-protein complex in Bt that is essential for growth on starch, uses eight proteins (SusRABCDEFG) to process starch. SusCDEFG localize in the outer membrane and likely form a complex to facilitate starch binding, degradation and import. However, conventional biochemical methods have been unable to completely reveal the assembly and dynamics of these proteins in response to starch. We have applied single-molecule super-resolution imaging to characterize the Sus complex response to different sugars in live Bt under anaerobic conditions. Protein correlation studies performed with HaloTag-labeled SusG and other fluorescent antibody-labeled Sus proteins demonstrated that simple sugars such as glucose or maltose do not induce Sus complex assembly. Conversely, incubation of Bt cells with starch enhanced the co-localization of Sus proteins, suggesting starch-induced assembly of the complex. Furthermore, single-molecule tracking revealed that in the absence of starch, SusG predominately moves in a fast diffusion mode corresponding to freely diffusing SusG. In contrast, two-color single-molecule experiments performed with fluorophore-labeled starch and SusG showed that starch confines the free diffusion of SusG and induces the assembly of the complex. Overall, our results suggest that starch catabolism involves dynamic interactions of Sus proteins, which assemble as a complex in the presence of starch in live cells.

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Orbital Tracking of Single Fluorescent Particles on a Commercial Confocal Microscope
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Single Particle Tracking (SPT) is a super-resolution technique used to determine the position of fluorescent particles with nanometer localization. The localization is generally obtained by analyzing the spatial distribution of fluorescence intensity emitted by the particle. In fact, the center of the distribution can be determined with an uncertainty which is much lower than the size of the distribution itself. In the orbital tracking method the position of a particle is obtained analyzing the distribution of intensity along a circular orbit scanned around the particle. In combination with an active feedback this method allows tracking of particles in 2D and 3D with millisecond temporal resolution[1]. More recently, the use of orbital tracking to perform imaging has also been proposed[2]. The orbital tracking and the other 3D SPT feedback methods are generally implemented on homebuilt microscopes which are not yet commercially available. On the other hand, commercial setups offer the advantage of a user-friendly software interface and pre-calibrated hardware components. It would be of interest to implement a SPT setup based on a feedback approach with minimal modification of a commercial available microscope. Here we explore this idea using a widely used confocal laser scanning microscope, the Zeiss LSM 510, in combination with an external piezoelectric stage scanner. We discuss advantages and limitations of this implementation of the orbital tracking method and the potential application to live cell experiments.

References:

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Single-Molecule Fluorescence Imaging of Reco Localization and Dynamics in Bacillus Subtilis
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In all organisms, the high fidelity of DNA replication is essential for maintenance of chromosome integrity. DNA damage can be caused by polymerase errors or by external factors (e.g., X-rays or mutagenic chemicals). Thus, the cell has evolved a number of repair mechanisms to respond to different types of damage. In B. subtilis, repair of double-strand breaks (DSBs) in the DNA occurs through RecA-mediated homologous recombination. The mechanism by which RecA finds DSBs in vivo is not well described, but it is believed to involve the proteins RecF, RecO, and RecR. This role for RecO in DSB repair, in B. subtilis is distinctly from its role in E. coli but analogous to that of Rad52 in eukaryotes, making B. subtilis an excellent model system for studying cellular response to DNA damage. Previously, bulk fluorescence studies have shown that RecO forms foci after the induction of double-strand breaks. However, RecO in undamaged cells can only be visualized when overexpressed, leaving questions about its true localization at wild type expression levels. Here, we have created cells in which PAmCherry-RecO is natively expressed from the RecO promoter as the only RecO source. We use single-molecule fluorescence microscopy in live B. subtilis to show that RecO rapidly forms foci following treatment with the DNA damaging agent phleomycin, but is diffuse throughout the cell under non-damaging conditions. This result suggests that, unlike several other proteins involved in DSB repair, RecO is not associated with the recombination process to DSB recognition. Future work will examine the previously reported role of single-strand binding protein (SSB) in recruitment of RecO to DSBs.

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Versatile Pulsed 560 nm Laser Source for Time-Resolved Microscopy and Spectroscopy
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Fluorophores have become a standard tool in life science and biophysics as well as material sciences. Nowadays, fluorophore position and concentration are often not sufficient to answer questions in fluorophore dynamics, such as diffusion behavior or molecular interactions. These aspects become accessible with the application of time-resolved fluorescence microscopy and...