Title
Phasor unmixing to reveal organelle organization and cellular response

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DNA structural transitions facilitate genomic processes, mediate drug-DNA interactions, and inform the development of emerging DNA-based biotechnology. Here, we use concurrent fluorescence polarization imaging and DNA manipulation experiments to probe the structure of DNA conformations that form under high tension and topological constraints. First, we employ polarization imaging of DNA intercalators (small dye molecules that slide between the DNA base-pairs) to probe the structure of S-DNA, an elongated conformation that can be accessed by mechanical overstretching of (relaxed) B-DNA. At extensions beyond the DNA overstretching transition, our polarization microscopy technique reveals that intercalators adopt a tilted orientation relative to the DNA axis, distinct from the nearly perpendicular orientation normally assumed at lower extensions. These results provide the first experimental evidence that S-DNA has substantially inclined base pairs relative to those of B-DNA. Next, we extend our method to investigate how topological constraints (DNA pulling geometry, torsional constraint, and negative supercoiling) influence the orientations of intercalated dyes. In contrast to earlier predictions, the pulling geometry (namely, whether the DNA molecule is stretched via opposite strands or the same strand) is found to have little influence. However, torsional constraint leads to a substantial reduction in intercalator tilting in overstretched DNA, particularly in AT-rich sequences. Surprisingly, the extent of intercalator tilting is similarly reduced when the DNA molecule is negatively supercoiled up to a critical supercoiling density corresponding to $\sim$70% reduction in the linking number. We attribute these observations to the presence of P-DNA (an overwound DNA conformation). Our results suggest that intercalated DNA preferentially flanks regions of P-DNA rather than those of S-DNA and substantiate previous suggestions that P-DNA forms predominantly in AT-rich sequences.

1344-Pos

Advanced microscope studies of the cell polarization instructing activity of Ras proteins

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Ras gene mutations are detected in around 30% of human cancers. These mutations have been linked with polarity disruption in epithelial cells, a hallmark for cancer. Still, the cell depolarization mechanism remains unknown. Here, we investigate polarized cells by means of advanced fluorescence microscopy and spectroscopy approaches, such as superresolution STED microscopy, fluorescence correlation spectroscopy, and spectral imaging, to explore the differences between apical and basal membranes regarding lipid organization and Ras-lipid interactions. Importantly, we overcome the difficulties to measure polarized membranes with light fluorescence microscopy due to the photoselection effect, i.e., the preferential excitation of membrane dyes depending on their orientation in respect to the excitation light polarization. For that, we propose three different approaches, namely, (i) 3D culture methods to modify membrane orientation, (ii) oblique light sheet illumination, and (iii) radial excitation light polarization. Our results are important to the study of polarized cell membranes using advanced fluorescence microscopy, and for the understanding of the role of Ras and its interaction with lipids.

1345-Pos

Oligomerization state of the functional bacterial twin arginine translocation (Tat) receptor complex

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The twin-arginine translocation (Tat) system transports folded proteins important for growth and survival across bacterial and plastid energy transducing membranes. Ion leaks are generally considered to be mitigated by the creation and destruction of the translocation conduit in a cargo-dependent manner, a mechanism that enables tight sealing around a wide range of cargo shapes and sizes. Since the Tat system is conserved among bacteria and absent in mammalian cells, it is an attractive target for antibacterial therapies. A minimal Tat system in *Escherichia coli* is comprised of the three proteins TatA, TatB, and TatC. The TatB and TatC proteins form a 1:1 complex, while TatA is in excess. Although the composition of the active Tat translocon is potentially variable due to cargo-dependent recruitment of TatA for transport, the receptor complex, which binds the Tat signal peptide, is more stable but has proved stubbornly difficult to establish. We analyzed the single molecule photobleaching characteristics of individual inverted membrane vesicles (IMVs) with Tat proteins tagged with the fluorescent protein mNeonGreen. These fluorescent IMVs, which act as individual reaction chambers with stochastically distributed Tat proteins, were immobilized on a coverslip. The step histograms determined from photobleaching traces were compared with the expectation values obtained by assuming that the Tat complexes were Poisson distributed into the IMVs with a binomial distribution of fluorescently active mNeonGreen proteins. The best fit indicates that Tat receptor complexes are tetrameric in native membranes with respect to both TatB and TatC. This establishes a maximal diameter for a resting state closed pore. A large percentage of Tat-deficient vesicles explains the typical low transport efficiencies observed. This individual reaction chamber approach will facilitate examination of the effects of stochastically distributed molecules yielding distinct transport behaviors.

1346-Pos

Phasor unmixing to reveal organelle organization and cellular response

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Organelles are a series of subcellular structures that perform various functions inside eukaryotic cells. The coordination between organelles play an important role in understanding the intricate biochemical processes within eukaryotic cells as organelles must work in concert to maintain cell function. Here, we perform simultaneous excitation of seven fluorophores and spectral phasor unmixing to achieve robust emission separation of all seven stained subcellular compartments and organelles including the Golgi apparatus, tubulin, lipid droplets, lysosomes, mitochondria, nuclear and mitochondrial DNA. Our imaging approach uses a single round of two-photon excitation at 780 nm coupled with multicolor organelle-directed staining to obtain multi-parametric physiological profiling of live MCF10A cells, a non-tumorigenic epithelial breast cancer cell line, under stress related treatments. We created an analysis pipeline to spectrally unmix the contribution of each organelle and obtain phenotypic signatures under each type of treatment. Ultimately, we characterized significant differences in organelle phenotypes upon application of a variety of treatments, including Antimycin A (mitochondrial respiration), hydrogen peroxide (oxidative stress), Nocodazole (microtubule depolarization) and serum starvation. The phasor approach provides a robust tool in separating up to seven simultaneous emission spectra and could be useful in separating even more components by complementing with lifetime imaging.

1347-Pos

Exploring cell-surface nanopillar interactions with 3D superresolution microscopy

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Plasma membrane topography has been shown to strongly influence the behavior of many cellular processes such as clathrin-mediated endocytosis (CME) and actin rearrangement. Recently, three-dimensional (3D) nanostructures such as nanopillars have been used to precisely control the local membrane curvatures of mammalian cells. In these studies, the behavior of cytoplasmic proteins at cell-nanopillar regions (the ''nano-bio interface'') were probed by conventional two-dimensional fluorescence microscopy. However, the low resolution and limited axial detail of such methods are not optimal to determine the relative spatial position and distribution of proteins along 100 nm-diameter objects, which are below the optical diffraction limit. Here, we introduce a general method to explore the nanoscale distribution of biomolecules along nanopillars with 10-20 nm precision using 3D single-molecule superresolution (SR) localization microscopy. This is achieved by combining a silicone-oil immersion objective (SIO) and double-helix point spread function (DHPSF) engineering methods. To minimize spherical aberrations introduced by the refractive index mismatch between quartz nanopillars and the cell, we first carefully optimize a silicone-oil objective with a correction collar. We validate the 3D SR method by imaging surface-labeled nanopillars using the SIO and a DHPSF microscope and compare the results with electron microscopy measurements. Turning to transmembrane-anchored labels in cells, the high quality 3D SR reconstructions show approximately a 50 nm gap between the membrane and the underlying nanopillar. Additionally, we find that the cytoplasmic protein AP-2, which is involved in CME, preferentially accumulates along the nanopillar above a threshold of 1/R (reciprocal of the radius) membrane curvature. Finally, we observe that both AP-2 and actin preferentially accumulate at positive Gaussian curvature near the pillar caps. Our results establish a new general method to investigate the nanoscale distribution of biomolecules at the nano-bio interface using 3D SR microscopy.