many laboratories will have most of their constructs in GFP form and entire genomes are available as functional GFP-fusion proteins.
Here, we report a method that makes all these constructs available for superresolution microscopy by targeting GFP with tiny, high-affinity antibodies coupled to blinking dyes. It thus combines the molecular specificity of genetic tagging with the high photon yield of organic dyes and minimal linkage error, as demonstrated on microtubules, living neurons and yeast cells. We show that in combination with GFP-libraries, virtually any known protein can immediately be used in superresolution microscopy and that high-throughput superresolution imaging using simplified labeling schemes is possible.
The labeling density in superresolution microscopy based on photoactivatable fluorophores is limited by the fact that a small, but significant fraction is always in the bright state. To overcome this limitation we implemented binding-activated localization microscopy (BALM), which is based on the localization of individual binding events of fluorophores that show a fluorescence enhancement upon binding to their target structures. Using nucleic acid stains on double-stranded DNA we yielded a resolution of ~14 nm (fwhm) and a spatial sampling of 1/nm in vitro and could visualize the organization of the bacterial chromosome in fixed Escherichia coli cells. In general, the principle of binding-activated localization microscopy can be extended to other dyes and targets such as protein structures.

51-Subg
Simultaneous Imaging of Vesicle Trafficking and Calcium-Mediated Exocytosis in Pancreatic Beta-Cells
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It has long been known that only a small fraction (<10%) of the insulin in pancreatic beta-cells can be released. Clinical treatments of type 2 diabetes focus on increasing insulin release, so an understanding of insulin vesicle trafficking and release may lead to novel therapeutic strategies. Towards this understanding, we have utilized a novel quantitative imaging assay, based on a double fusion to the integral vesicle protein phogrin. In this construct, a pH-dependent ecliptic pHluorinFP is inside the lumen of the granule and a mOrangeFP is outside the granule in the cytoplasm. The pHluorin fluorescence is quenched by the low pH inside the granule until exocytosis when the lumen of the granule mixes with the extracellular media. This increases the local pH and the green fluorescence can then be measured as a readout of exocytosis. Experiments were done on the multicolor TIRF system with a photoactivation module. We photoconverted the mOrange-phogrin to a deep red protein in the perinuclear region, and then watched where that population of granules went as we stimulated the cells with glucose. Having only a small subset of vesicles labeled greatly facilitates tracking. We used this approach with TIRF microscopy to measure secreted granules and determine if they came from previously docked granules or from regions beyond the TIRF imaging field. In beta-cells, we find that secreted vesicles do not come from a docked pool, with secretion happening within about 1 second of the vesicle's arrival at the membrane. This time is decreased even further with the addition of glucose. These data suggest that the releasable insulin pool in beta-cells may not be docked at the membrane as are synaptic vesicles, but rather are maintained deeper with in the cell.

52-Subg
Invasive Optics for Watching the Brain Work
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Optical methods are revolutionizing neuroscience, from high-resolution multiphoton microscopy, to functional imaging with Ca^{2+}- and voltage-sensitive dyes, to direct optical stimulation and inhibition of neurons with light. Yet this incredibly powerful toolbox has been limited in its application by an inability of microscopy to image deep in the living brain. We have made great strides in this direction through novel invasive micro-optics capable of penetrating the brain to gain access to deep brain regions. We will show recent results, including simultaneous Ca^{2+}-sensitive imaging of hundreds of neurons throughout all 6 layers of cortex in awake mice.

53-Subg
Assembly and Dynamics of Nucleic Acid - Protein Complexes at the Single-Molecule Level
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Single-molecule fluorescence spectroscopy is emerging as a powerful tool for detailed biophysical analyses of nucleic acid - protein interactions, because of the ability to resolve different binding modes and to provide kinetic information on protein conformational changes during assembly or biological function. To illustrate these capabilities, I will describe two systems currently under study in my laboratory. (1) The HIV-1 protein Rev mediates the nuclear export of unspliced and partially spliced mRNAs encoding viral structural proteins. Rev interacts with a highly conserved element within the viral pre-mRNA known as the Rev response element (RRE). Multiple Rev monomers must assemble on the RRE, mediated by a combination of RNA-protein and protein-protein interactions. Multi-color single-molecule TIRF microscopy is used to monitor hundreds of individual Rev-RRE assembly reactions in parallel, revealing the mechanism of oligomeric assembly and the influence of cellular cofactors on the assembly pathway. (2) DNA polymerases replicate DNA substrates with extraordinarily high fidelity because of their ability to discriminate between cognate and non-cognate nucleotide substrates during each cycle of nucleotide incorporation and to remove misincorporated bases using a separate proofreading activity. Single-molecule FRET methods are used to observe conformational changes of the fingers subdomain of a model DNA polymerase during the process of selection of incoming nucleotide substrates, revealing a novel ''ajar'' conformation that acts as a fidelity checkpoint before the fingers enclose the nascent base pair. These methods are also used to monitor the movement of the nascent DNA strand during proofreading, revealing that the DNA can switch between the spatially separated polymerase and exonuclease sites while remaining bound to the enzyme. These observations provide new insights into the important role of enzyme conformational dynamics during the processes of nucleotide substrate selection and proofreading.

Subgroup: Membrane Structure & Assembly

54-Subg
Discovering Highly Potent Pore-Forming Peptides using Synthetic Molecular Evolution
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There are many natural and designed peptides that permeabilize membranes, and there are multiple mechanisms by which membrane permeabilization can occur. Yet, peptides that unequivocally self-assemble into equilibrium, membrane-spanning pores at low peptide to lipid ratios (P:L1:1000) are very rare. The design and engineering of such peptide ''nanopores'' in lipid bilayer membranes is desirable as it could lead to improved biosensor platforms, targeted therapeutics, exogenous ion channels, or drug delivery vehicles. While the few well studied pore-forming peptides have provided a lot of information about the architecture of peptide pores, especially -helical pores, our knowledge of the fundamental molecular principles of pore formation is not detailed enough for rational engineering. This is a roadblock to the design of new pore-forming peptides and to the optimization of known pore-formers for particular applications. In this work we show how novel, highly potent, equilibrium pore-forming peptides can be discovered using synthetic molecular evolution, i.e. iterative cycles of combinatorial library design and high-throughput screening. In the first example, we used two generations of *de novo* library design and screening to identify highly potent pore-formers that self-assemble into -sheets in membranes. These peptides may be the only known examples of highly potent, pore-forming peptides that have -sheet secondary structure in membranes. In the second example we designed an iterative library that used the helical pore-former melittin as a template. From this library we identified gain-of-function pore-formers that are much more potent than melittin. The results demonstrate the power of synthetic molecular evolution for the discovery and engineering of membrane active peptides.

55-Subg
Exploring the Mechanisms of Antimicrobial Lipopeptides with Molecular Simulation
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Antimicrobial peptides (AMPs) — small peptides that kill bacteria and fungi by attacking their outer membranes — have been touted as a possible solution to the emergence of new strains of antibiotic-resistant pathogens. Although some AMPs have become promising drug candidates, overall they have not been especially successful clinically, primarily because of their size and bioavailability. To avoid these difficulties, we have focused our efforts on smaller peptides chemically modified to include acyl chains at their N-termini; the fatty acid chains give the peptides the ability to bind membranes efficiently without including a large number of hydrophobic side chains. Using a combination of