quantitative fluorescence microscopy and cryo-electron microscopy, have become a powerful approach to extract the underlying rules of how the microtubule cytoskeleton acts as a dynamic system. For example, through this approach the hierarchical functioning of a regulatory protein interaction network at growing microtubule ends, formed around so-called end binding (EB) proteins, can now be understood from the atomic to the micrometer scale. In the future, the challenge will be to reconstitute even more complex systems to be able to test directly our understanding of higher-order cytoskeletal functions.

1125-Symp
The Forces that Center the Mitotic Spindle
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Precise positioning of the mitotic spindle is important for specifying the plane of cell division and the subsequent partitioning of the cell's contents to the daughter cells. Studies on different organisms and cell types have suggested diverse centering mechanisms: astral microtubules grow out from the spindle and push against the cortex, cortical dynein motors pull on astral microtubules, and dynein-dependent organelle transport on astral microtubules leads to a reactive force on the spindle. The different mechanisms lead to different predictions for the precision of centering, how mutations effect the precision, and the magnitude of the forces associated with spindle centering. We used image processing to accurately track the position and orientation of the mitotic spindle during the first cell division in the C. elegans embryo. The high precision of centering, < 1% of cell diameter transverse to the anterior-posterior axis, increased after RNAi against gpr-1/2, genes encoding activators of the cortical force generators; this suggests that centering is not mediated by gpr-1/2-dependent cortical pulling forces. To measure the forces associated with spindle positioning, we built a magnetic tweezers apparatus so that forces could be exerted on the spindle via beads incorporated into the embryo: forces of approximately 20 pN were required to displace the spindle through 1 μm. These mechanical experiments constrain molecular models of the centering process.

Platform: Imaging & Optical Microscopy: Superresolution Imaging & Single Molecules

1126-Plat
Sub-100 nm 3D Detection Volumes by Total Internal Reflection STED Microscopy
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Stimulated Emission Depletion (STED) Microscopy (1–2) has revolutionized far-field fluorescence microscopy by breaking the classical diffraction limit: 25 nm resolution and better are routinely achieved in the focal plane.

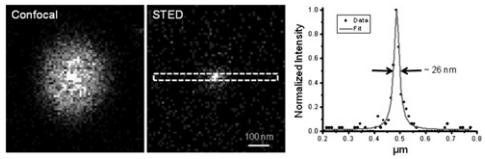

Fig. 1. 20 nm fluorescent bead imaged in confocal and STED mode. The profile across the white box demonstrates <26 nm resolution.

While comparable axial resolution values have been obtained using two opposing objectives , single-objective STED microscopy can usually not realize values on this size scale.
Here we present results obtained with a recently realized STED variant that combines total internal reflection excitation for ~70 nm axial sectioning capabilities with stimulated depletion for ~50 nm lateral super-resolution.
TIRF STED microscopy represents an attractive super-resolution alternative for live cell microscopy featuring fast scanning with sub-100 nm 3D detection volumes and reduced photo-damage through TIRF excitation.
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1127-Plat
Super-Resolution dSTORM Imaging of Human Galectin-1 Interacting with Neuroblastoma Cells
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Galectins are a family of carbohydrate-binding proteins with an affinity for beta-galactosides. They share a core sequence consisting of 130 amino acids, and the beta-sandwich fold. Human Galectin-1 (hGal-1) is a well studied representative of prototype galectins, non-covalently linked homodimers with two specific carbohydrate recognition domains (CRD). It is differentially expressed by various normal and pathological tissues and is involved in intra- and extracellular processes like cell adhesion, formation of galectin-glycoprotein lattices, signal transduction and regulating immune responses, inflammation, allergies, and host-pathogen interactions. Furthermore oxidized galectins are associated with the regeneration of the central nervous system after injury.
We use direct stochastic optical reconstruction microscopy (dSTORM) to study the spatial organization of hGal-1 interacting with glycans like ganglioside GM1 presented on the membrane of human SK-N-MC neuroblastoma cells. Using the photoswitchable fluorophore ALEXA 647 as specific galectin marker, we employ fluorescence on/off switching with standard widefield microscopy and spot analysis of single molecules in order to resolve clustering, localization, and cross-linking of galectins on the cell surface with a spatial resolution of less than 50 nm.
We study spatial organization and its dependence on galectin concentration and oxidation state, as well as inhibition of the specific recognition.

1128-Plat
Direct Live-Cell Super-Resolution Imaging of Cellular DNA
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Direct stochastic optical reconstruction microscopy (dSTORM) is an imaging method that relies on the stochastic photoswitching of single fluorophores and enables resolution of structures down to tens of nanometers in biological samples. Recently, several proteins have been visualized with dSTORM in live cells by using genetically encoded tags labeled with chemical dyes, including DNA-associating proteins. However, despite its importance in cellular processes, live-cell super-resolution imaging of DNA structure itself has never been demonstrated. We present the imaging of DNA with dSTORM based on direct DNA labeling. We optimized buffer conditions to achieve the reversible photoswitching required for dSTORM in living cells and used it to resolve nuclear and mitochondrial DNA structures. Furthermore, due to the excellent preservation of the dyes, we were able to perform time-lapse super-resolution imaging. This illustrates that is possible to monitor the sub-diffraction limited organization of DNA in individual cells over time.
This protocol in combination with protein super-resolution imaging provides an advantageous tool to study processes related to DNA dynamic structural rearrangements such as those occurring during cell division or in response to cell stress.

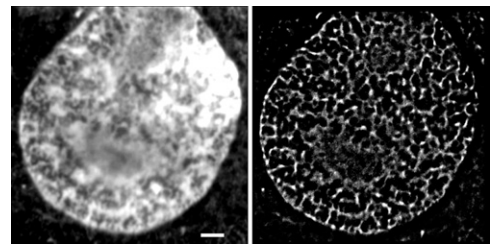
Figure: Wide-field (left) and dSTORM (left) images of cellular nucleus. Scale bar 2.5μm.

1129-Plat
Live Cell Super-Resolution Imaging of Transverse Membrane Tubules in Heart Failure
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Transverse tubules (TTs) are hollow intracellular membrane structures, coupling action potential propagation at the cell surface to intracellular

Ca^{2+} release sites in cardiomyocytes. Since individual TT structures are below the resolution limit of convential light microscopes, the nanoarchitecture in living heart cells is uncertain. Using STED (stimulated emission depletion) nanoscopy and the membrane probe di-8-ANEPPS, we characterized TTs quantitatively deep inside living cardiomyocytes. Using 2D analysis of TT cross-sections we determined diameters and contours indicating regular cylindrical structures. However, after myocardial infarction (post-MI) TT cross-sections appeared dilated. In addition, the continuous TT network changed progressively, resulting in loss of regular tubule orientations and sarcomere misalignement 8 weeks post-MI. In contrast, 4 weeks post-MI a signficant increase of longitudinal network components was evidenced, resembling an immature embryonic phenotype. Analysis of TT associated protein expression confirmed embryonic remodeling 4 weeks post-MI. Furthermore, mathematical modeling of increased spacing between TTs and intracellular Ca^{2+} release channels revealed delayed subcellular Ca^{2+} release and action potential prolongation in heart failure. In conclusion, STED nanoscopy of intracellular TT structures in living myocytes provides direct insight about a fundamental mechanisms of excitation-contraction coupling and how TT changes contribute to Ca^{2+} release dysfunction in heart failure.

1130-Plat

Superresolution and Single Molecule Imaging of Transcription by Reflected Light Sheet Microscopy

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Understanding gene expression in mammalian cells requires techniques that allow to probe abundance, dynamics and distribution of the key molecular players such as transcription factors, RNA Polymerase and RNA inside the nucleus. To circumvent ensemble averaging of biomolecular dynamics, single molecule sensitivity inside the cell is essential, while superresolution imaging beyond the diffraction limit is needed to resolve the structural architecture of macromolecular assemblies involved in transcription.

We achieve both high temporal and spatial resolution in single cells using a new variant of light sheet microscopy. This new technique, Reflected Light Sheet Microscopy, employs a small mirror near the cell to project a light sheet into the nucleus. This optical sectioning reduces out-of-focus background and allows video-rate detection of individual fluorescent proteins and superresolved STORM imaging in mammalian nuclei.

In live cells, tracking of single transcription factors labeled with a fluorescent protein reveals the kinetics of free diffusion as well as non specific and specific binding to DNA. Furthermore, STORM imaging in fixed cells using fluorescently labeled antibodies against different phosphorylated forms of RNA polymerase II reveals a homogenous distribution of transcription throughout the nucleus. This superresolution view of mammalian transcription provides no evidence for transcription factories.

1131-Plat

Live-Cell Super-Resolution Fluorescence Imaging at High Spatiotemporal Resolutions

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Super-resolution fluorescence microscopy accomplished nanometer resolutions in optical imaging along with multi-color and three-dimensional capability in fixed specimens. For live cells, time and spatial resolutions has been limited by the intrinsic tradeoff between the two resolutions. Using bright, fast switching cyanine dyes, we achieved high spatiotemporal resolutions with stochastic reconstruction microscopy (STORM). We obtained two-dimensional (2D) and three-dimensional (3D) super-resolution images of clathrin-coated pits and their transferrin cargo labeled with photoswitchable dyes either directly or via SNAP tags in living cells. The high photon output and fast switching rate of the cyanine dye enabled us to demonstrate 2D imaging at spatial resolutions of ~25 nm and temporal resolutions as fast as 0.5 sec, which represent a 2-3 fold improvement in spatial resolution and 50-100 fold improvement in temporal resolution compared to previously reported resolutions obtained using single-molecule-localization based super-resolution approaches. Furthermore, we demonstrate live-cell 3D volumetric super-resolution imaging for the first time, with a spatial resolution of ~30 nm in the lateral directions and ~50 nm in the axial direction at time resolutions down to ~1 sec. Two-color 3D super-resolution imaging in live cells was also achieved using photoswitchable probes with distinct emission spectra. In addition, we obtained similarly high spatial and temporal resolutions imaging other important cellular structures. These imaging capabilities open a new window for resolving ultrastructures in living cells.

1132-Plat

2D/3D Super-Resolution Microscopy by Means of Individual Molecule Localization (IML) and Selective Plane Illumination Microscopy

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In the last few years localization based techniques, which exploit photoactivation, photoconversion or ground state depletion of fluorescent molecules, became a popular tool for super-resolution imaging of biological samples. Recently, approaches based on two photon excitation have been implemented in order to get axial confinement of the photoactivation process allowing for 3D super-resolution imaging of biological samples [1]. However, a topic of interest is still represented by the widening of super-resolution applications to thick samples (>15 μm). Within this scenario, light sheet based fluorescence microscopy techniques provide optical sectioning since illumination is confined to a thin planar region perpendicular to the detection axis and represent a suitable tool to confine the photoactivation process along the optical axis. In particular, single plane illumination microscopy (SPIM), has been proved to be a useful tool for biological investigations of thick living samples [2]. Here we demonstrate three-dimensional super-resolution live cell imaging through thick biological specimen (>50 μm), by coupling far-field individual molecule localization (IML) and selective plane illumination microscopy (SPIM).

The confined excitation provided by SPIM and the improved signal-to-noise ratio allows for nanometric localization of single molecules in thick scattering samples. A PALM approach [3] and elliptical stretching of the point spread function allow to perform 3D super-resolution imaging of biological live samples in depth (up to 100 μm). IML-SPIM allowed to image cellular spheroids with < 35 nm lateral precision and sub-diffraction resolution in depth [4].

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1133-Plat

STED - AFM: Tip Probing Enhanced by Super Resolved Targeting

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Tip based topological studies are always very dependable when we question minute structures like proteins or behaviors like aggregation, adhesion, conformational or morphological changes. Dedicated tip based microscopy, as atomic force microscopy; revealed new insights in biology and medicine in the past decades with the new ability of ''touch and manipulate'' objects at the nanoscale. Besides, AFM based methods solved many functional queries of the scientific community. Here, we show the enhanced ability of pointing AFM on a region of interest by super resolved imaging of a fluorescent population by coupling it to a far-field optical nanoscopy method, STED microscopy. We report about the first realization of AFM-STED hybrid architecture. We demonstrate AFM-STED measurements on various samples of scientific pursuit with a precision better than 60 nm. This new overlay of optical images of fluorescent structures to AFM surface studies; enables the system with the fundamental element of accuracy and helps in directing the tip to any region of interest. This is particularly well suited system, for example: for membrane dynamics studies and for detecting mechanically induced stress effects on precisely defined regions at the nanometer resolution. We think that this opens an important window on the design of new brand experiments in biophysics at the nanoscale.

Platform: Protein Dynamics

1134-Plat

Dynamics at Specific Sites in Proteins Studied by 2D IR Vibrational Echo Spectroscopy

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The techniques of two-dimensional infrared (2D IR) spectroscopy enable the study of fluctuations of molecules and their environments on fast timescales. Characterization of specific sites in proteins requires the use of extrinsic vibration probes to alleviate the spectral complexity that hinders the application of IR spectroscopy with protein samples. Heme-bound CO has been a popular vibrational probe of protein active sites and, for example, was employed to characterize the involvement