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Quantitative Registration and Distribution Analysis of Multicolor 3D Super-Resolution Images of Proteins Reveals Nanoscale Spatial Organization
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The subcellular localization of biomolecules within cells is of crucial importance to signaling and cell cycle regulation. Super-resolution fluorescence microscopy enables determination of molecular locations with 20-40 nm precision, allowing for observation of previously invisible spatial organization. However, to conclusively determine whether biomolecules labeled with different colors colocalize at this length scale, the color channels must be overlaid with an accuracy $\leq$ 10 nm. Furthermore, to extract quantitative information about biomolecular spatial organization from single-molecule localizations, unbiased analytical methods that account for the discrete and stochastic nature of localization-based imaging methods are essential.
Here, we demonstrate a method to obtain quantitatively overlaid multicolor three-dimensional super-resolution images. We apply these capabilities to study the unknown spatial organization of proteins at the ~250 nm diameter cell poles of the bacterium *Caulobacter crescentus*. We describe algorithms that analyze 3D single-molecule localization data within this small space by determining overlap and morphological similarity between protein spatial distributions. We can thus map out the three dimensional architecture of proteins at the cell pole and determine the average distances between protein clusters with nm precision.

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Direct Three-Dimensional Imaging with Multiple Point of View Microscopy
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In traditional fluorescence microscopy, the sample is imaged in the focal plane of the objective. In order to obtain a three-dimensional view of a fluorescent sample, one has to record multiple parallel planes and combine them to create a three-dimensional reconstruction of the sample. This can be too slow to allow recording of the dynamics of the process of interested and is fundamentally limited by a low axial resolution. These limitations do not allow dynamics quantification of three-dimensional processes below typically a few seconds timescale.
We present a new technique, Multiple Point of View Microscopy that allows the simultaneous recording of two or more planes in the sample with independent orientation and position. By means of micro-mirrors and recently developed refocusing techniques, direct three-dimensional imaging is obtained, not limited in time resolution and with substantially improved axial resolution compared to traditional fluorescence microscopy. We demonstrate the power of this approach in experiments on intraflagellar transport in Caenorhabditis elegans. We show that we can track the translocation of GFP-labeled kinesins in vivo in three dimensions at video rate.
This demonstrates that Multiple Point of View Microscopy can be used for three-dimensional quantification of dynamics taking place in three dimensions on virtually any fluorescent sample, ranging from single molecules to multicellular organisms.

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Mitochondrial DNA Nucleoid Distribution at Simulated Pathologies as Visualized by 3D Super-Resolution Biplane FPALM / dSTORM Microscopy
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Upon certain insults and/or pathological states fragmented mitochondrial (mt) network persists in cells, with spheroid mt remnants containing clustered nucleoids of mtDNA (1).
To image nucleoid distribution within mt network, we employed 3D super-resolution fluorescent photoactivable localization microscopy (FPALM)(2), in conjunction with photoconvertible fluorescent protein conjugates of marker proteins such as mitochondrial (mt) transcription factor A (TFAM) or mt single-stranded-DNA-binding protein (mtSSB). We have shown that nucleoids in hepatocellular HepG2 cells exhibit a rather wide size distribution ranging between 50 to 300 nm. This was confirmed using 3D TFAM/mtSSB immunocytochemistry in conjunction with direct stochastic optical reconstruction microscopy (dSTORM). Positioning in TIM23 dSTORM visualized membranes have confirmed our previous model for nucleoid clustering in mt network fragments (1), which were induced by mtDNA replication/transcription inhibition or other pathogenic simulations.
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Optofluidic Single-Cell Rotation
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The combination of optics and microfluidics is a very powerful and non invasive tool to perform single cell analysis and imaging. Being able to measure properties at a single cell level is of fundamental importance to understanding cellular systems and their heterogeneity. We present flow cytometry systems with added rotation techniques to gain isotropic high resolution 3D images of the measured objects. They can be combined with most optical microscopy systems and used with a wide range of fluorescent dyes and antibody staining. The optofluidic cell rotator (OFCR) holds single live cells in suspension using a dual beam optical trap and can rotate the cells by either manipulating the microfluidic flow or the alignment of the trapping beams. Such manipulations allow in plane, as well as tomographic rotation of the cell. By monitoring the cell during the smooth and controlled tomographic cell rotation, we can reconstruct a 3D image of the cell with an isotropic resolution, bypassing the normally lower z-resolution. Additionally, by measuring the nature of the rotation itself, it is also possible to observe underlying properties of the cells.
We have explored a number of methods to perform cell rotation. One straightforward possibility is to flow liquid past the trapped cell. If the cell is held in the correct position, the imbalance of the forces acting across the cell surface results in a smooth tomographic rotation. We are able to show by cell rotation that the z-resolution can be improved by threefold over confocal imaging of the same cell. Another parameter which can be controlled is the relative position of the optical fibres, leading to a fibre-optic spanner. We have developed a novel dynamic fibre-optic spanner, capable of detailed measurement of the rotation properties of cells within the trap.

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Polarized Resolved Single-Molecule Localization-Based Super-Resolution Fluorescence Microscopy Reveals Orientation Order in Bio-Molecular Assemblies
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Fluorescence microscopy allows noninvasive imaging of numerous species in biological specimens with single-molecule sensitivity. Super-resolution microscopy techniques based on single molecules localization, taking advantage of this sensitivity, have brought a significant improvement in nano-scale imaging of molecular assemblies in biological specimens. These methods can now image living cells [2-3], three-dimensional specimens [4], and multiple species. However, apart from one early demonstration [5], these methods are not exploited to provide information on the orientation and rotational freedom of individual molecules, which can be used to analyze the degree of interaction between molecules in biological systems. Furthermore, understanding organization and functionality of molecular machines often requires the determination of the orientation of molecules within cellular structures and the relation between one molecule and its surrounding ones.
In this work, we report a super-resolution polarization-resolved microscopy technique able to image molecular orientation in static and dynamic environments at single molecule level and nano-scale resolution. Using direct Stochastic Optical Reconstruction Microscopy (dSTORM) [6] in combination with polarized detection, fluorescence anisotropy images can be reconstructed at a spatial resolution of 30nm, from which both the dynamical nature of the molecular orientational order and its static angular constraint can be deduced. Based on a refined signal analysis technique which provides high accuracy in the estimation of molecular anisotropies, we report nano-scale orientational behaviors of biomolecular assemblies in fixed cells and *in vitro*, such as in amyloid aggregates and actin fibers.
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