Mechanism of Polyene Antibiotics

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The FAS complex is manipulated for the production of non-native products. A relatively simple model of their interplay can explain the observed product sites, respectively responsible for elongation and termination, and establish that of relative enzyme-substrate binding free energies. We focus on two key active enzymes.

atomistic molecular modelling, molecular dynamics simulation and calculation of enzymatic systems producing almost exclusively palmitic (C16) and stearic (C18) acids.

Here we consider the fatty acid synthase (FAS), a 2.6MDa multi-enzyme complex consisting of six heterodimers, which catalyses nine separate reactions successively repeated reaction steps, each extending the growing acyl chain by two carbon atoms. Chain length is precisely controlled, with eukaryotic metabolic systems producing almost exclusively palmitic (C16) and stearic (C18) acids.

We here consider the FAS complex and the enzyme IXa.

of eight distinct types of active site. The high local concentrations resulting from this arrangement, further facilitated by an inter-domain ‘shuttle’ domain, enable more efficient processing than could be achieved with freely diffusing enzymes.

To elucidate how the extremely tight control of chain length is achieved from the perspective of the underlying molecular recognition processes, we used atomistic molecular modelling, molecular dynamics simulation and calculation of relative enzyme-substrate binding free energies. We focus on two key active sites, respectively responsible for elongation and termination, and establish that a relatively simple model of their interplay can explain the observed product spectrum, and that our method correctly predicts changes in chain length arising from tested mutations.

These findings will direct ongoing work in which the synthetic machinery of the FAS complex is manipulated for the production of non-native products. The developed methods will contribute to techniques available for rational enzyme engineering.

Aggregation States Driven by Dipole Interactions Regulates Action Mechanism of Polyene Antibiotics

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1, 2, 3, 4, 5, 6

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Super resolution microscopy techniques such as fluorescence photo-activation localization microscopy (FPALM), photo-activation localization microscopy (PALM), and stochastic optical reconstruction microscopy (STORM) have revolutionized biological microscopy by allowing an increase in final image resolution by a factor of ten over traditional diffraction limited microscopy. Cells are labeled with photo-activatable/switchable fluorescent molecules which become visible to the observer when illuminated with a laser. Phase separation between two wavelengths of laser is required in order to obtain super-resolution images. This requires slow, repetitive photo-stimulation of molecules and has enabled the determination of the orientation of individual proteins within samples, the tracking of individual proteins in living samples, the imaging of proteins in three dimensions, and the distinction of multiple fluorescent species. Here, we focus on two-dimensional super resolution imaging. Previous super-resolution techniques have relied upon splitting the fluorescence signal from multiple species into channels of different wavelength bandwidths using dichroic mirrors; the ratio of intensities between these channels provides the means of species distinction. Alternatively, interchanging multiple excitation lasers can excite different fluorescent species at different time periods. Here, we present an alternative method for super-resolution FPALM multispecies imaging. We use a combination of optics to measure the spectral distribution of single molecule fluorescence. In this way we can distinguish multiple distinct fluorescent species. We have applied this technique successfully to obtain super-resolution FPALM images, visualizing multiple photo-activatable fusion proteins simultaneously within NIH-3T3 fibroblast cells.

Accurate EMCCD Photoelectron Calibration for Single Molecule Imaging Techniques

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EMCCDs are widely used in super-resolution and single molecule imaging methods. These techniques rely on photon counts for particle detection and calculating localization precision of single molecules, making accurate calibration of EMCCD camera counts to photon counts necessary. Calibration methods used in literature include shot noise based calibration and manufacturer-provided formulas, but there is no general consensus and the accuracy of these methods has yet to be verified. First, we directly measure the light response of an EMCCD using a photon counting approach. We use a greatly attenuated laser to illuminate a few pixels on an EMCCD and compare the FCS data points to the readout of a photon counting photomultiplier tube placed in the same position. Second, we process uniformly illuminated EMCCDs images to isolate shot noise as the dominant noise source to create an adjusted mean-variance method of calibrating EMCCDs. We verify this adjusted mean-variance method using our photon counting approach. Third, we compare these results to the theoretical values from the EMCCD calibration methods used in literature. To test our modified calibration method, we examine single molecule localization data and compared the data to existing models.

Accurate EMCCD Photoelectron Calibration for Single Molecule Imaging Techniques

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Super resolution microscopy techniques such as fluorescence photo-activation localization microscopy (FPALM), photo-activation localization microscopy (PALM), and stochastic optical reconstruction microscopy (STORM) have revolutionized biological microscopy by allowing an increase in final image resolution by a factor of ten over traditional diffraction limited microscopy. Cells are labeled with photo-activatable/switchable fluorescent molecules which become visible to the observer when illuminated with a laser. Phase separation between two wavelengths of laser is required in order to obtain super-resolution images. This requires slow, repetitive photo-stimulation of molecules and has enabled the determination of the orientation of individual proteins within samples, the tracking of individual proteins in living samples, the imaging of proteins in three dimensions, and the distinction of multiple fluorescent species. Here, we focus on multispecies imaging. Previous super-resolution multicolor techniques have relied upon splitting the fluorescence signal from multiple species into channels of different wavelength bandwidths using dichroic mirrors; the ratio of intensities between these channels provides the means of species distinction. Alternatively, interchanging multiple excitation lasers can excite different fluorescent species at different time periods. Here, we present an alternative method for super-resolution FPALM multispecies imaging. We use a combination of optics to measure the spectral distribution of single molecule fluorescence. In this way we can distinguish multiple distinct fluorescent species. We have applied this technique successfully to obtain super-resolution FPALM images, visualizing multiple photo-activatable fusion proteins simultaneously within NIH-3T3 fibroblast cells.

Accurate EMCCD Photoelectron Calibration for Single Molecule Imaging Techniques

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Single molecule imaging is a useful tool for confirming protein-protein interactions using small sample volumes. Here we present our studies of antibody binding to antigen captured on a surface using fluorescently labeled antigen and antibody, using total internal reflection fluorescence microscopy we
show the kinetics of antigen and antibody binding in real-time without significant contribution of signal from background fluorescence. We also show how single molecule analysis allows determination of the labeling efficiency of the antibody bound to the surface. By analyzing the bleaching steps of individual fluorophores at a given location, we can determine the number of dye molecules attached to randomly labeled antibody conjugates. Our data using this method indicates a bias towards antibody labeled with less fluorophores. Finally, we show single molecule detection of sub-picomolar concentrations of antigen using well-characterized antibody reagents.

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3D-Super-Resolution Microscopy Reveals MRNA Nano-Structure in Stress Granule

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We investigated these issues by stochastic optical reconstruction microscopy (STORM), which provides us super-resolution images with spatial resolution of ~20 nm in the lateral direction and of ~60 nm in the axial direction. Furthermore, we performed three-dimensional super-resolution imaging using cylindrical lens. To visualize endogenous cytoplasmic mRNAs, we microinjected Cy5-labeled linear antisense 2'-O-methyl probes into the cytoplasm of COS7 cells. After the injection, cellular stress was induced by addition of 0.5 mM arsenite in a culture medium. To investigate the maturation of SGs, STORM images were captured at various time-points during SG formation.

Three-dimensional super-resolution images showed that endogenous mRNAs located in spherical compartments with a diameter of ~200 nm. Since these compartments were densely packed within several micrometers radius, we could not observe these structures by diffraction-limited imaging. We termed this structure “mini-granule”. With stress duration, mini-granules increased in number, while they maintained the same size. These data demonstrated that the growing process of SGs resulted from the assembly of mini-granules. The result of this study indicated that mini-granules were responsible for the physiological functions of SGs.

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Crossing the Border towards Deep UV Time-Resolved Microscopy of Native Fluophores

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More than 20 years ago, single photon counting based techniques evolved as one recognized standard in fluorescence detection. In combination with confocal microscopy FLIM (Fluorescence Lifetime Imaging Microscopy) and FCS (Fluorescence Correlation Spectroscopy) became established techniques for investigations down to the single molecule level. Up to date, these experiments typically are carried out in the visible up to the near infrared spectral range. Based on recent advances in fiber amplified laser technology [1] and ultrasmall detection, we present a novel approach to extend time-correlated single photon counting (TCSPC) into the deep UV using 266 nm excitation. Hereby, direct access is granted to the native fluorescence of biomolecules originating from appropriate chromophoric groups such as the amino acids tryptophan and tyrosine within proteins. As first results, we will present label-free FLIM of cells where the aromatic amino acids within the proteins become visible. As a benchmark, also FCS with organic fluorophores in the deep UV will be shown.

Another application of time-resolved fluorescence microscopy in the deep UV includes microfluidics and thus enables label-free detection and identification of various aromatic analytes in chip electrophoresis [2, 3]. Fluorescence decay curves are gathered on-the-fly and average lifetimes can be determined for different substances in the electropherogram with the aim to identify aromatic compounds in mixtures. Based on the time-correlated single photon counting the background fluorescence can be discriminated resulting in improved signal-to-noise ratios. In addition, microchip electrophoretic separations with fluorescence lifetime detection can be performed with protein mixtures emphasizing the potential for biopsy analysis.

References:
[1] Schoenau et al., Biomedical Optics (BIOMED), Miami, Florida, 2012

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Localization Precision for Asymmetric Single-Molecule Images in Superresolution Localization Microscopy

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We present theoretical localization precision formulae for asymmetric single-molecule images in superresolution localization microscopy. Superresolution localization microscopy, such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), have demonstrated superior performances in cell imaging and enable the investigation of cellular processes at close to the molecular scale. All these techniques rely on the precise localization measurements of single-molecules at the nanoscale by using statistical estimators to fit diffraction-limited single-molecule images with the theoretical point spread function (PSF) of the imaging system, which is commonly approximated as a two-dimensional Gaussian. However, to our best knowledge, all previous theories [e.g., R. E. Thompson et al, Biophys. J. 82, 2775 (2002) and R. J. Ober et al, Biophys. J. 86, 1185 (2004)] on theoretical localization precision are developed for circular symmetric single-molecule images. In contrast, many of the recent advances in the developments of localization microscopy have demonstrated that astigmatism can occur and result in asymmetric PSFs as a result of optical aberrations in the imaging system [S. Ohla et al, Proc. Natl. Acad. Sci. USA 2012, 109, 675 (2012)] and asymmetric molecular emission [K. I. Mortensen et al, Nat Meth 7, 377 (2010)]. Asymmetric PSF has also been implemented in localization microscopy to achieve astigmat imaging for three-dimensional single-molecule localization techniques [B. Huang et al., Science 319, 810 (2008)]. Therefore, our new theories for asymmetric single-molecule images can be particularly useful where asymmetric PSFs have been used or observed in localization microscopy.

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3D STED Microscopy in Scattering Specimens

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By breaking the classical diffraction limit, Stimulated Emission Depletion (STED) Microscopy has revolutionized far-field fluorescence microscopy. 25 nm resolution and better have been achieved in two dimensions imaging cultured cells and even neurons in the brain of living mice. 3D super-resolution has also been demonstrated utilizing two opposing objectives or phase filters with a top-hat profile (see Figure), its application to tissue has however been hampered by aberrations introduced by refractive index inhomogeneities.

Here we present our latest results in 3D STED microscopy of scattering specimens enabled by the integration of adaptive optics into a custom STED microscope. We will present our current research about the physical and technical concepts of adaptive optics STED microscopy as well as the latest biological applications of adaptive optics STED microscopy.

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STED Microscopy with Time-Gated Detection: Benefits and Limitations

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In a stimulated emission depletion (STED) microscope the region in which fluorescence markers can emit spontaneously shrinks with continued STED beam action after a singular excitation event. This fact has been recently used [1] to substantially improve the effective spatial resolution in STED nanoscopy using pulsed excitation, continuous wave (CW) STED beams and by sorting photons depending by them arrival-times (time-gated detection). We present theoretical experimental data that characterize the time evolution of the effective detection volume of a STED microscope and illustrate the physical basis, the benefits, and the limitations of this new STED implementation, namely gated CW-STED (gCW-STED). Among the STED implementations, gCW-STED provides the highest effective resolution at low light intensity and is in essence limited (only) by the reduction of the signal that is associated with gating. Time-gated detection also strongly reduces the influence of local variations of the fluorescence lifetime on STED microscopy.