MEASURING THE PHASE AND INTENSITY OF THE LIGHT: COMPLEMENTARY INFORMATION FOR BIOLOGICAL SAMPLE CHARACTERIZATION AT THE NANOSCALE.

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KEY WORDS: 3D single molecule localization, quantitative measurements, phase imaging, interferometry, deep tissue

Conventional optical imaging technique are only sensitive to the light intensity. However, many other optical parameters can be probed to enhance the quantity of information retrieved from a biological sample. I will discuss in this presentation how and why measuring the so-called phase of the light. Applications both in the scope of label-free microscopy and for 3D fluorescence super-resolution will be discussed. In particular, I will show that this quantitative imaging modality allows to identify without labeling many organelles at high frame-rate and for any duration¹ in living cells; this approach can be easily combined with single molecule studies in dynamic samples. Then I will move to the application of imaging both the intensity and the phase of fluorescent single emitter and demonstrate that it leads to single molecule 3D localization and 3D super-resolution² and single particle tracking³ even at depth in biological tissues.



Figure 1: (Left) Quantitative phase imaging of label-free human fibroblasts. (Right) 3D super-resolution image reconstruction using quantitative phase and intensity measurement of f-actin.

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Measuring differential diffusion rates of distinct protein states by sptPALM imaging in live microbial cells

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Correct spatiotemporal organization is essential for many cellular processes and the formation of protein gradients plays a significant role in establishing specific intracellular localization patterns. With the help of sptPALM imaging, we can measure the spatial organization and dynamics of single molecules in live microbial cells at high specificity, sensitivity and spatiotemporal resolution. In particular, it enables us to measure the individual molecules in the heterogeneous cellular environment where we can observe their behavior in presence of the other components of the cellular machinery. This distinguishes sptPALM experiments from *in vitro* single-molecule assays and their very controlled environment in test tubes.

By using sptPALM imaging, we thus can follow the localization dynamics and differential diffusion rates of distinct conformational protein states which drive and regulate intracellular pattern formation. In this talk, I will introduce some of our recently developed experimental and analytical sptPALM tools alongside with our specific microbiological questions.

Switchable fluorophores – perspectives for advanced imaging

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Keywords: super-resolution microscopy, switchable fluorophores, fluorogenic probes, live-cell microscopy

Observation and photo-physical control of single molecules have enabled advanced approaches in fluorescence microscopy with improved sensitivity and resolution. Established techniques, like single-molecule localization microscopy, now call for improved fluorophores opening the field for diverse contributions from synthetic chemistry. Over the past years, we have been working on switchable fluorescent probes controlled by chemical equilibrium and demonstrated their use for single-molecule localization microscopy, alternative multiplexing, and sensing. [1,2,3] Here, we will present novel fluorogenic probes for use in advanced imaging of living cells based on the known tetrazine-based click chemistry. We studied the mechanism of the click reaction with different substrates by use of single-molecule methods where we could identify different fluorescent species in course of the reaction. We have also implemented selfblinking fluorophores by use of spiro-cyclization to enable live-cell single-molecule localization microscopy where we found extended observation times. This work shows the potential of different quenching and reaction mechanisms for advanced imaging emphasizing the importance of contributions from synthetic chemistry.

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MINFLUX Nanoscopy: Superresolution post Nobel

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The 2014 Nobel Prize in Chemistry was awarded "for the development of super-resolved fluorescence microscopy". More than 125 years after Ernst Abbe's definition of the supposedly insurmountable diffraction resolution limit, fluorescence "microscopes crossed the threshold", as the Nobel poster put it. The result has been the breathtaking development of far-field optical super-resolution microscopy or, in short, 'nanoscopy' as an entire field over the past years.

A fresh look at the foundations [1] shows that an in-depth description of the basic principles of nanoscopy spawns new powerful concepts such as MINFIELD [2], MINFLUX [3] and DyMIN [4]. Although they differ in some aspects, these concepts harness a local intensity minimum (of a doughnut or a standing wave) for determining the coordinate of the fluorophore(s) to be registered. Most strikingly, by using an intensity minimum of the excitation light to establish the fluorophore position, MINFLUX nanoscopy has obtained the ultimate (super)resolution: the size of a molecule [3]. The talk will highlight recent developments.

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Spectral Cross-Cumulants for Multicolor Super-resolved SOFI Imaging

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Super-resolution optical fluctuation imaging (SOFI [1]) provides a resolution beyond the diffraction limit by computing higher-order statistics of a time series (few hundred to thousands frames) of independent, stochastically blinking fluorophores. Using n^{th} order spatio-temporal cross-cumulants the spatial resolution as well as the sampling can be increased up to *n*-fold in all three spatial dimensions. In this study, we extend the cumulant analysis into the spectral domain and propose a novel multicolor super-resolution scheme. The simultaneous acquisition of two spectral channels followed by spectral cross-cumulant analysis and unmixing increases the spectral sampling. Besides a reduced acquisition time compared to sequential imaging, our analysis allows to unmix several fluorophore species even with strongly overlapping emission spectra, where the number of species is not limited to the number of physical spectral channels. Using two color channels, we demonstrate spectral unmixing of three fluorophore species in simulations and multiple experiments with different cellular structures, fluorophores and filter sets. We also provide a guideline for optimized spectral filter choice based on an eigenvalue/ vector analysis. The possibility of cross-cumulating between color channels thus translates the concept of spatial super-resolution to spectral super-sampling. Our analysis preserves all the advantages inherent to SOFI such as optical sectioning, elimination of uncorrelated background and increased spatial resolution. Since we formulated multicolor spectral cross-cumulant SOFI in the theoretical framework originally devised for spatially super-resolved SOFI, it is intrinsically compatible with previous developments such as 3D SOFI [2] and bSOFI [3]. Overall, our methodology provides a novel route for easy-to-implement multicolor subdiffraction imaging using standard microscopes. This makes simultaneous multiplexed superresolution fluorescence imaging widely accessible to the life science community interested to probe colocalization between two or more molecular species.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant Agreement No. 750528.

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Combining advanced microscopy techniques for fast 3D imaging at the single molecule level in living cells

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Keywords: 3D live imaging, Single molecule localization and tracking, Light sheet, Nucleus

Fluorescence microscopy is a fundamental tool in modern biological studies. Single molecule imaging, localization and tracking allow to investigate the nanoscale organization and dynamics of biomolecules in their cellular environment with high temporal and spatial resolution. However, conventional single molecule imaging systems are limited in the imaging depth and cannot consider the inherent and complex 3D organization of cells. Moreover, efficient 3D single particle tracking requires high temporal resolution and sensitivity over an extended volume. Finally, it is fundamental to confine the excitation to match the detection volume and reduce the background noise. In fact, widefiled excitation could degrade single molecules detection efficiency and increase photo-toxicity and photo-bleaching. To address these challenges, we combined two cutting-edge microscopy techniques: MultiFocus Microscopy (MFM) [1] and single objective Selective Plane Illumination Microscopy (soSPIM) [2].

MFM produces instantaneous volumetric imaging without any sequential mechanical scanning. By simultaneously acquiring different focal planes of the biological specimen with a single camera, MFM allows the fluorescence detection over an axially extended volume with a high temporal resolution only limited by the acquisition speed of the camera. The high temporal

resolution of MFM dramatically extends the observable range of single molecule dynamics.

To confine the excitation beam, soSPIM architecture uses a single high numerical aperture objective to create a light sheet and collect the emitted fluorescence thanks to a mirror places at 45° angle on the coverglass beside the sample. soSPIM strongly reduces the out-of-focus signal, allowing to detect single molecules at several microns inside the sample.

Here, we demonstrate that the combination of soSPIM and MFM enables to image a 3D volume of few microns axial extent with a time resolution down to 30 ms and single molecule sensitivity (FIG. 1). We believe that the proposed combination paves the way for exciting investigation of biological functions where spatial and temporal resolutions are limiting factors.



FIG. 1 – MFM image of nucleopore complex labeled with GFP obtained using soSPIM excitation confined to 6 planes.

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Visualization and quantification of plasma membrane receptors by super resolution microscopy

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Keywords: dSTORM, receptor quantification, lattice light-sheet, expansion microscopy

Receptors hold a prominent role as inductors and receivers of signals in cells. They are essential for pathways including apoptosis and proliferation [1].

Visualization of the entire plasma membrane "Receptome" of a cell remains challenging and quantitative information about receptor distributions on cell membranes is largely missing, despite its huge potential impact on targeted medical treatments. Due to its explicit single molecule sensitivity, super resolution microscopy by *d*STORM enables the analysis of the plasma membrane receptors with molecular resolution. [2] While multi-color *d*STORM approaches are limited by the amount of spectrally distinguishable dyes, we aim to quantify several receptors by sequential imaging with the optimal *d*STORM dye Alexa Fluor 647 using Exchange-*d*STORM [3].

To visualize a whole T-cell at single molecule sensitivity we applied lattice light-sheet (LLS) *d*STORM [4] and 10x expansion microscopy [5], revealing for most receptors a homogeneous receptor distribution in the plasma membrane.



Abbildung 1 3D-dSTORM-LLS image of CD3 enables visualization of a whole Jurkat T-cell identifying a homogeneous receptor distribution on the coverslip.

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How do BRCA2 molecules find DNA damage in nuclear space?

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Keywords: single-molecule, tracking, live cell, DNA repair

For fundamental understanding on how proteins exert their function at the right time and place it is important to have an accurate description of their movement within cells. With singlemolecule tracking it is possible to obtain this information from living cells and link the diffusive behavior of proteins to their function. We investigated the mobility of the tumor suppressor BRCA2, which is a versatile multidomain protein, with several roles in maintaining genomic stability in most eukaryotes. While present at a low concentration, BRCA2 needs to act specifically at sites of DNA damage, which are distributed across the nuclear volume. Thus, changes in nuclear location and accumulation are essential fundamental aspects of its function.

To quantify BRCA2 movement in live cells we generated mouse ES cells expressing endogenous BRCA2 fused with HaloTag, which allows for conjugation of bright fluorescent dyes such as JF549 to BRCA2 inside living cells. The individual molecules can be followed up to several seconds, providing detailed information about diffusion and transient immobilization. Upon induction of DNA damage by different reagents we observed increased immobilization of BRCA2 molecules in cells. In order to analyze the mobility of BRCA2 at sites of damage specifically, we induced local DNA damage in subnuclear regions. At these sites of damage, we observe slow and confined motion of BRCA2 at sub compartments surrounding the DNA damage. Furthermore, we quantified the spatial organization of BRCA2 and other DNA repair proteins surrounding the break using single-molecule localization microscopy (dual-color STORM). The nanoscale analysis of the mobility and localization of DNA repair proteins will help in understanding what molecular transactions are required to properly repair the DNA damage.

Super-Resolution Spatiotemporal Mapping of DNA Double Strand Break Repair

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Keywords: DNA damage, DNA double strand breaks, dSTORM

DNA damage response (DDR) pathways are involved in both the cause and potential treatment of various cancers, auto-immune, and neurodegenerative diseases. Our current understanding of DDR has been elucidated over the course of several decades by combining biochemical and biophysical techniques, however, imaging of damage and repair in vivo has remained challenging. This has predominantly been because of the dense and varied nature of the nuclear environment, and the diffraction limit of light. Here, we have successfully used dSTORM super resolution (SR) imaging to circumvent this limit and capture spatially and temporally resolved snapshots of double strand break (DSB) repair in cells [1]. Moreover, we specifically generated individual single-ended DSBs similar to those endogenously created by collapsing replication forks. The resulting repair foci could be visualized in multicolor SR by labelling nascent DNA via modified base incorporation and click chemistry, DSBs via the TUNEL assay or direct ligation, single stranded DNA via BrdU incorporation, and proteins via immunolabelling. The enhanced spatial and temporal resolutions and the singular nature of the DSBs themselves revealed several exciting and novel insights including the dynamic interactions of proteins such as Ku, MRE11 and RAD51 at the DSB, the redundant role of RAD52 in repair, and a critical in vivo BRCA2 dependence on BRCA1 [2]. We have also extended these assays to investigate the roles of long non-coding RNA [3] and novel proteins [4] in DSB repair. I will present these findings within the context of their importance to the genomic integrity research community, as well as the broader novelty and applicability of the SR assays we have developed.

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Time modulated illumination for 3D single molecule localization microscopy

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In Single Molecule Localization Microscopy (SMLM), the position of the emitter is obtained by fitting the Point Spread Function (PSF). This spatially based localization precision thus strongly relies on the PSF shape, which is degraded by defocusing or aberrations. We propose a new localization strategy based on a time signature to retrieve simultaneously over the whole field of view the position of the fluorophores. Uniform excitation is replaced by a shifting structured excitation over the entire field of view, typically using a moving fringe pattern. This induces a time modulated emission of the illuminated fluorophores whose phase shift encodes its position. The modulation frequency much be carefully chosen to be compatible with the short ON-time of single molecule emitters (typically shorter than 20 ms in dSTORM) and its Poissonian statistics. Modulation frequencies typically higher than 500 Hz are needed, which is too high to be detected with most cameras in full frame acquisition mode. We have designed a dedicated optical assembly placed in front of the camera to perform fast demodulation of all emitters simultaneously. This device uses a Pockels cell to steer the photons in 4 sub-images acquired in a single camera frame. Demodulation is achieved without any photon loss while being compatible with photophysics of most emitters. The assets and performances of this new localization technique called ModLoc for Modulated Localization, will be discussed, in particular its 2-fold improvement of the lateral localization precision compared with PSF fitting. We will also present an implementation of ModLoc for an improved axial localization in 3D SMLM. The modulation is applied in a tilted direction through the objective. An almost isotropic 3D localization (~11 nm) is obtained over the whole capture range, and for depths up to 30 µm. Several biological applications will be presented, as an example figure1 represents a ModLoc image of clathrin coated pits above the nucleus of COS7 cells.





Fig. 1 A) COS7 cell with clathrin labeled with AF 647, imaged above the nucleus.B) Close-up corresponding to the whiteboxed region in A).

Uniting Structured Illumination and Localization Microscopy (SIMFLUX)

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KEY WORDS: super-resolution microscopy, SIM, localization microscopy

MINFLUX offers a breakthrough in single molecule localization precision, but suffers from a tiny field of-view and a lack of practical parallelism. SIMFLUX makes use of extended illumination patterns similar to those used in Structured Illumination Microscopy (SIM). Here, we combine centroid estimation and illumination pattern induced photon count variations in a conventional widefield imaging setup to extract position information over a typical micron sized field-of-view. We show a near twofold improvement in precision over standard localization microscopy with the same photon count on DNA-origami nano-structures and Tubulin, imaged with PAINT and dSTORM.



Figure 1 | Principle of SIMFLUX. A total of 6 images are recorded with 3 shifted patterns per orthogonal orientation of the line pattern. Combining the centroid estimates of the 6 frames with the photon count in relation to the pattern shift improves the localization precision with a factor of around two compared to the standard centroid estimate on the sum of the 6 frames.

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The characterization of the spatial-temporal dynamics of Aquaporin-2 using photoactivated localization microscopy and spatiotemporal image correlation spectroscopy

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Keywords: sptPALM, STICS, AQP2

Aquaporin-2 (AQP2) is an important membrane protein in the water-body homeostasis. AQP2 is a homo-tetrameric water channel found in kidney collecting duct cells (MDCK cells) located in intracellular vesicles in the cell cytoplasm. Upon phosphorylation the protein is inserted in the membrane, where it enables the water to flow through the membrane to concentrate the urine [1]. To study the membrane protein organization and nanoscale dynamics, single particle tracking photoactivated localization microscopy (sptPALM) has been utilized. The sptPALM dataset is then analyzed with spatiotemporal imagine correlation spectroscopy (STICS). STICs uses a filtering mechanism to remove frequencies associated with immobile components, allowing measurements of protein dynamics even in the presence of a large fraction of immobile species [2].



Figure 1: **Imaging and analysis of AQP2 in the cell membrane.** Representative PALM image of MDCK cells expressing AQP2 (A), representative STICS vector map of AQP2 velocity in the membrane (B).

Using sptPALM and STICS, we acquired transport and spatial maps of AQP2 molecules in the kidney cell plasma membrane (Figure 1). Forskolin is used in the experiment to activate adenylyl cyclase and increase concentration of cAMP, which leads to phosphorylation and insertion of AQP2 in the membrane. Analysis of velocity vector revealed a significant increased velocity of the AQP2 in the membrane upon forskolin treatment. These observations agrees with previous studies, where a higher velocity of AQP2 in the membrane correlated with phosphorylation level [3]. Our results provide the first look at the broad, nanoscale organization and dynamics of AQP2 in kidney cells, providing better understanding of AQP2 function in the kidney.

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Improving DNA-PAINT for the visualization of nuclear target molecules

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Keywords: DNA-PAINT, super-resolution, single-molecule localization microscopy, cell nucleus

DNA points accumulation in nanoscale topography (DNA-PAINT) is a single-molecule localization microscopy (SMLM) technique that is gaining popularity because of its ease-to-use. DNA-PAINT utilizes the transient binding of a fluorescently labeled oligo (the 'imager') with its complementary oligo ('binder'), which creates the 'blinking' events necessary for super-resolution microscopy. The programmability and specificity of the DNA oligo hybridization interaction enables the decoupling of the 'blinking' events from the photophysical properties of the single-molecule dyes, enabling the usage of DNA-PAINT for a wide variety of buffer conditions and dyes and as a quantitative super-resolution technique. Nevertheless, the employment of DNA-PAINT in environments where there naturally is DNA available, such as the nucleus of cells, might be hampered. Here, we characterize the unwanted binding events of imagers to the genetic DNA and offer a solution to circumvent these by using left-handed DNA for DNA-PAINT. We show that left-handed DNA-PAINT complies the same advantages for SMLM, but performs better in visualizing and quantifying nuclear structures.

Dense three dimensional localization microscopy by deep learning

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Keywords: deep learning, localization microscopy, 3D, PSF engineering

In localization microscopy, the positions of individual nanoscale point emitters (e.g. fluorescent molecules) are determined at high precision from their point-spread functions (PSFs). This enables highly precise single/multiple-particle-tracking, as well as super-resolution microscopy, namely single molecule localization microscopy (SMLM). Localization in three-dimensions (3D) can be performed by modifying the PSF using additional optical elements, e.g. a phase mask in the back focal plane of the microscope. Then, extracting the 3D position of the emitter from such an image is achievable using various parameter esitmation techniques, that typically perform very well for isolated emitters. However, localizing multiple adjacent emitters in 3D poses a significant algorithmic challenge, due to the lateral overlap of their PSFs.

Here, we train a neural net to receive an image containing densely overlapping PSFs of multiple emitters over a large axial range, and output a list of their 3D positions (Fig. 1). Furthermore, we then use the net to *design* the optimal PSF for the dense multi-emitter case. We demonstrate our approach numerically as well as experimentally by volumetrically imaging dozens of fluorescently-labeled telomeres occupying a mammalian nucleus in a single snapshot [1].



Fig. 1 A convolutional neural network is trained to receive a highly ovelapping image of multiple 3D encoding PSFs, and output their positions on a 3D grid. Blue circles indicate ground truth, red dots indicate recunstructed positions (simulation).

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Teaching deep neural networks to localize sources in super-resolution microscopy by combining simulation-based learning and unsupervised learning

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Keywords: High density localization, Deep learning

We present a novel deep learning based single molecule localization algorithm (DECODE) which significantly improves upon the state of the art [1]. Our contributions are a novel deep neural network architecture for simultaneous detection and localization, and a new training algorithm which enables this deep network to solve the Bayesian inverse problem of detecting and localizing single molecules.

Our DECODE network uses temporal context from multiple sequentially imaged frames to improve the detection and localization of single molecules by integrating information across time. Our training algorithm combines simulation-based supervised learning with autoencoderbased unsupervised learning to improve the robustness of predictions to imperfect knowledge of the point spread function, imaging noise characteristics and background fluorescence.

We demonstrate the performance of our method on simulated and real datasets with a variety of point spread functions and fluorophore densities. While existing localization algorithms can achieve optimal localization accuracy in data at low fluorophore density, they are confounded by high densities. Our method significantly outperforms the state of the art at high fluorophore density and thus enables faster imaging than previous approaches. Our work also more generally shows how to train deep networks to solve challenging Bayesian inverse problems in biology and physics.



Fig 1: Performance comparison on SMLM2016 challenge datasets at two different fluorophore densities and SNR levels.

DECODE achieves the highest fluorophore detection accuracy (Jaccard index) in 11/12 datasets, and the lowest localization error (RMSE) in 7/12. On the combined "efficiency" measure DECODE leads in all 12 datasets. Each marker indicates one benchmarked algorithm, large solid markers indicate DECODE.

Acknowledgements: This work was supported by the German Research Foundation (DFG) through SFB 1089, the German Federal Ministry of Education and Research (BMBF, project `ADMIMEM', FKZ 01IS18052 A-D), and the Howard Hughes Medical Institute.

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SMLM with light sheet illumination for deep cell imaging

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Single molecule localization microscopy methods like PALM [1] and STORM [2] allow to overcome the optical diffraction limit by a factor of ~10. However, these methods are limited to imaging a single focal plane in the sample because they generally use widefield or TIRF illumination. In TIRF, only the first hundred nanometers above the coverslip are imaged while in widefield deeper focal plane imaging is permitted but the out of focus planes are photobleached preventing their imaging in high resolution. In Single Plane Illumination microscopy (SPIM) [3], a single focal plane is illuminated by a thin light sheet, thereby leaving the out of focus planes free of photobleaching. Scanning the light sheet then enables volumetric imaging of the cell with a confocal resolution.

Taking advantage of both methods, and following previous work, we aimed to perform SMLM using a thin light sheet illumination in order to achieve volumetric high resolution microscopy of entire cells [4, 5, 6]. We present the development of a SPIM system for SMLM, which allows to perform high resolution imaging deeper in the cells. The microscope is based on a commercial body using 2 high numerical aperture and high magnification objective lenses oriented at 120° from each other. This configuration allows to create a very thin light sheet and to optimize photon collection [7]. We will show preliminary applications of this system to imaging nuclear pores and mitochondria at high resolution up to $\sim 25 \,\mu m$ in depth.



Figure 1: Images of nuclear pores with the oblique SPIM microscope. a) Low resolution image of three nuclei stained with NUP96-SNAP A647. b) High resolution image of the top nucleus. c) Zoomed view of the region in the white dashed rectangle in (b) showing single nuclear pore complexes.

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Mechanistic investigation of the photoconvertible fluorescent protein mEos4b reveals a strategy to increase tracklength in single particle tracking PALM.

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Keywords: fluorescent proteins, photoblinking, sptPALM

Fluorescent proteins (FPs) are widely used as genetically-encoded markers in practically all fluorescence microscopy schemes. Notably, a large toolbox of phototransformable fluorescent proteins (PTFPs) have been engineered in recent years, of use in many advanced modalities such as super-resolution imaging. PTFPs display a variety of photoactivation, photoconversion, photoswitching, photoblinking and photobleaching properties that can be used at advantage in many cases, but that are also at the origin of major complications and artifacts, making these fascinating "smart" labels still far from ideal and quite poorly understood mechanistically. In this work [1] we studied mEos4b, the latest variant in a series of highly popular green-to-red photoconvertible fluorescent proteins known to repeatedly enter dark states. These dark states cause, amongst other problems, interrupted tracks in single-particle-tracking localization microscopy (sptPALM), significantly compromising the huge potential of this technique for measuring complex diffusion patterns of proteins in live cells. Using a combination of kinetic crystallography under spectroscopic control and single-molecule measurements, we identified a long-lived dark state in photoconverted mEos4b and managed to trap it in crystallo to solve its 3D structure. We found that the dark state results from a light-induced "frustrated" cis-trans isomerization of the chromophore. In the trans configuration, the dark chromophore was observed to efficiently absorb light at 488 nm. This finding suggested that addition of weak 488-nm light could swiftly revert the dark state to the fluorescent state. This prediction was experimentally verified in the context of sptPALM on a microtubule associated protein (MAP4), opening the door to a very simple strategy to largely eliminate slow blinking and enable the recording of long single-particle tracks.



Fig: MAP4-mEos4b single-particle trajectories longer than 1 s in the absence and presence of 488nm light. The number of long tracks in the presence of 488 nm light is more than one order of magnitude higher.

 $(4.80 \text{ W} / \text{cm}^2)$

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Haste makes waste – Slow imaging increases resolution and labeling efficiencies in single-molecule localization microscopy

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Keywords: SMLM, imaging speed, resolution, optimization

Single-molecule localization microscopy (SMLM) is a slow super-resolution technique, as it requires acquisition of thousands of frames per image. To increase throughput, microscopists like to use high excitation intensities [1], which were believed to not strongly impair the image quality [2].

We set this believe on trial and found that both the effective labeling efficiency as well as the number of photons per localization strongly depend on the excitation intensity. Unconventionally low intensities of less than 1 kW/cm² result in more than 20,000 photons per localization for Alexa 647, and, hence, localization precisions on the order of one nanometer. Consequently, we could easily resolve individual molecules in the nuclear pore complex labeled by Alexa 647 dyes [3], which are only 11 nm apart. This takes dSTORM to the regime of DNA-PAINT [4]. While DNA-PAINT suffers from high backgrounds which restrict achievable precisions and probes which are not readily commercially available, both downsides are overcome by our approach.

Our findings also have a consequence for fast imaging. When low intensities instead of high intensities [5] are used for the initial off-switching, the labeling efficiencies are increased by a factor of two, even for fast imaging with high laser powers during data acquisition.

Applying the same pipeline to the photoconvertible fluorescent protein mMaple (PALM approach), we found a similar trend of decreasing photon counts with higher intensities. However, mMaple could still be localized at precisions better than 10 nm when imaging at 300 frames per second, allowing for fast live-cell PALM.

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Figure: Imaging speeds affect image quality in SMLM. Nup96-SNAP labeled with BG-AF647 in GLOX/BME buffer for exposure times between 500 ms (left) and 0.4 ms (right).

Parameter-free image resolution estimation based on decorrelation analysis

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Keywords: microscopy, resolution

Super-resolution microscopy opened diverse novel research directions by overcoming the classical resolution limit. Revealing structures beyond the diffraction limit was made possible by exploiting the fluorescent emission of individual fluorophores. However, accurately determining the resolution achieved during imaging is a challenging task. One approach consists in imaging structures of very well defined dimension such as nanorulers or nuclear pore complexes, providing an upper bound estimate of the resolution in controlled conditions. Another approach, known as Fourier Ring Correlation, relies on the acquisition of two consecutive images of the same object. Under the assumption of stationarity, FRC is able to provide an estimate of the in-situ resolution. However, the stationarity assumption is often challenged in real imaging settings where bleaching, drift and sample movement are unavoidable.

Here, we propose a method for assessing the resolution of individual microscopy images, superresolved or diffraction limited, based on image partial phase auto-correlation. The algorithm is model-free, does not require any user-defined parameters and enable real time assessment of the resolution without adding any constrains on the data acquisition scheme. We first demonstrate its validity by analysing DNA origamis and then illustrate its broad applicability by applying the method to various imaging modalities such as STED, SIM, SOFI and Localization Microscopy. We also discuss the interpretation, use and limitation of the method. Finally, we introduce an open source ImageJ plugin implementing the algorithm, enabling the use of the algorithm to non-image processing specialists.

Extracting molecular numbers from Photoactivated Localization Microscopy (PALM) data

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The function of proteins is often linked to their oligomeric state. Quantifying proteins within tiny membrane complexes in the cell remains a challenge with today's super-resolution methods. Here, we used quantitative PALM based on analysis of fluorescent protein blinking cycles using a stochastic model^[1,2]. First, we evaluated various photoactivatable fluorescent proteins and identified mEos2, mEos3.2 and mMaple3 as suitable for quantification with our approach. Second, we generated synthetic and genetic dimers as calibration standards, and determined bleaching and detection probability^[3]. Third, we demonstrated quantitative PALM in the cell membrane for protein complexes with known stoichiometry^[1,3], and interrogated protein complexes in the context of signaling^[4]. Finally, we support quantitative PALM analysis by an open source software^[5].

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Imaging single-molecules with temporal DNA barcodes

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Keywords: DNA kinetics, Optical multiplexing, Temporal patterns, Machine learning

Many biochemical events of importance are complex and dynamic. Fluorescence microscopy offers a versatile solution to study the dynamics of biology at the mesoscale. An important challenge in the field is the simultaneous study of several objects of interest, referred to as optical multiplexing. For improved multiplexing, some prior techniques used repeated reporter washing or the geometry of nanostructures; however, these techniques may require complex nanostructure assembly, multiple reporters or advanced multistep drift correction. Here we propose a time-based approach, for improved optical multiplexing, that uses readily available inexpensive reporters and requires minimal preparation efforts. We program short DNA strands, referred hereby as DNA devices, such that they undergo unique conformation changes in the presence of the dye-labeled reporters. The *universal fluorescent reporter* transiently binds with the devices to report their activity. Since each device is programmed to exhibit different hybridization kinetics, their fluorescent time trace, referred to as the temporal barcode, will be unique. We model our devices using Continuous-time Markov Chains and use stochastic simulation algorithm to generate their temporal patterns. We first ran several simulation experiments with a small number of our devices, demonstrating several distinct temporal barcodes, all of which use a single dye color [1]. Later, using a nanostructure, we designed a much larger pool of unique temporal barcodes and performed supervised learning using support vector machine [2]. Our simulation experiments and design principles can aid and influence the experimental design of temporal DNA barcodes. Using these learning, we experimentally demonstrate eight different devices with three tunable device parameters using TIRF microscopy [3].

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Linking nano- and meso-scale compartmentalization of the plasma membrane using high density single particle tracking tools

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Organization by compartmentalization is a general property of natural systems that efficiently facilitates and orchestrates biological events in space and time. In the last decade, compartmentalization of the plasma membrane of living cells has emerged as a dominant feature present at different spatiotemporal scales and regulating key cell functions. The advent of super-resolution microscopy and single molecule dynamic approaches has allowed the study of the cell membrane with unprecedented levels of details. From these studies it is becoming clear that receptor nanoclustering prior to ligand presentation constitutes a functional working unit of mammalian cells, including those of the immune system. In this talk, I will focus on recent studies in my group aiming at linking spatial and temporal organization of the cell membrane at the nano- and meso-scales. For this, we combine multicolor single particle tracking approaches at different labelling densities. Low density conditions allow us to reconstruct the mobility of individual receptors and their transient interaction with other molecular partners, while high density labeling conditions provide complementary information on the spatial and temporal length scales of membrane regions re-visited (or forbidden) for receptors. In particular, I will show recent data on the adhesion receptor CD44, a highly glycosylated receptor that connects the extracellular glycocalyx matrix to the intracellular actin cytoskeleton. Our results reveal a hierarchical organization of CD44 at multiple spatiotemporal scales, which is dependent on the turnover dynamics of the underlying cortical actin cytoskeleton.

A single-molecule, super-resolution view on intracellular transport in living *C. elegans*

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Our cells and in particular our neurons are too large for thermal-driven diffusion to be an effective means of transport of proteins, organelles and other cargoes. Instead, eukaryotic organisms have evolved motor proteins that drive unidirectional motion over relatively long distances while consuming chemical energy in the form of ATP. Over the last two decades, important insights into the molecular mechanisms of several key motor proteins of the kinesin, myosin and dynein families have been obtained, among others by the application of advanced single-molecule methods. Over the last years, the focus in my laboratory has shifted from studying the behavior of single motor proteins, working on their own *in vitro*, to studying motor proteins in their cellular environment. In our cells, motor proteins do not work on their own: cargoes are often transported by multiple motors, of the same type, but often also of opposite directionality. In addition, our cells are a very crowded environment, with many proteins bound to the motors' tracks, which might hamper their motion and could lead to 'traffic jams'.

To study these kinds of problems, we have focused on a particular transport mechanism, intraflagellar transport (IFT), which takes place in cilia and flagella and is essential for the assembly and maintenance of these organelles. As a model system we use IFT in the chemosensory cilia of the nematode *C. elegans.* In these organisms, IFT is driven by groups of tens of three different motor proteins: 2 kinesin-2's (the slow kinesin-II and the fast OSM-3) that drive transport of cargo trains from base to tip of the cilium, and IFT dynein that drives transport back to the base. In order to visualize IFT components with fluorescence microscopy, we generate mutant-nematodes expressing fluorescent versions of the IFT component of interest. Our fluorescence and image analysis approaches allow us to visualize, track and quantify trains of IFT components moving together as well as individual motor or IFT proteins. Single-molecule trajectories allow us to construct super-resolution maps of the cilia. Together, bulk and single-molecule data provide new, deep insights into the mechanisms of motor cooperation and cargo delivery, which we try to capture in quantitative models.

4Pi SMLM and the 3D architecture of the Nuclear Pore Complex

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Super-resolution localization microscopy techniques such as PALM and STORM enable the visualization and quantification of sub-cellular structures at the nanometer scale. Interferometric fluorescence detection, in particular, yields the highest possible localization precision along the Z dimension, allowing for fluorescence microscopy datasets with isotropic 3D image resolution of 10 nm or better. At this level of detail, it becomes possible to visualize the sub-structure of protein complexes, and we have applied this approach to study Nuclear Pore Complex (NPC) organization. The combination of genetically engineered cell lines, small fluorescent tags, super-resolution 3D imaging, and particle alignment methods adjusted for localization microscopy data, allowed us to obtain multicolor reconstructions of NPCs which may be directly compared with cryo-electron microscopy structures. Our approach allows for the alignment and visualization of an arbitrary number of distinct structural elements in the NPC, and these results represent the first example of multicolor 3D light microscopy of a single protein complex at 10 nm resolution. Furthermore, as the number of aligned particles increases, individual fluorescent tags within the NPC structure may be localized with arbitrarily high precision. Finally, the specificity and high contrast of the fluorescence data allows new insights into NPC biology which are not accessible by other methods, and our work represents a further step in the development of light microscopy as a tool for structural biology studies.

Uniform and versatile illumination method for Single Molecule Localization Microscopy

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Keywords: TIRF, SMLM, wide field of view, uniform, versatile, scanning

In SMLM and wide-field microscopy in general, lasers with gaussian-shaped illuminations are used and induce non-uniform excitation at the sample plane. In SMLM, this results in field-dependant photon count, localization precision, on-time... and hinders quantitative analysis of fluorescence processes. Strategies have been developed to provide wide-field uniform illuminations. Among others, fibres[1], micro-lens arrays[2] or refractive beam-shaping elements[3] effectively provide uniform excitation but their implementation tends to prevent their application to TIRF excitation and are often constrained to a fixed field of view (FOV) size. For SMLM, where 5 to 10kW/cm² excitation power is needed to ensure single molecule regime, it leads to the use of high 4-5W power to cover a wide FOV (200µm)² imaging.

We will present an illumination technique which is based on a classical 300mW, 647nm laser and benefits from the flexibility of a scanning-mirror system to achieve uniform illumination over the $(200\mu m)^2$ FOV of a sCMOS camera. By focusing the laser on the scanning mirrors, we can control its position in the sample plane and uniformly spread laser power on any FOV. This hybrid scanning/wide field excitation can be applied from TIRF to epifluorescence excitation [Fig. 1]. In SMLM, this approach allows us to control the sectioning and improve the signal to noise ratio. Furthermore, this novel excitation setup can also enhance photons counts and perform a trade-off between localization precision and blinking density. For example, this allows super-resolution imaging of mitochondria at the whole cell level and at low laser power [Fig. 2].



Figure 1 : EPI (cyan) and TIRF (red) uniform imaging of COS7 microtubules labelled with AF647.

Figure 2 : Wide-field SMLM image of Mitochondria achieved with 200mW effective laser power.

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Super-resolution fight club: Assessment of 2D & 3D single-molecule localization microscopy software

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Keywords: SMLM, image processing

With the widespread uptake of 2D and 3D single molecule localization microscopy, a large set of different data analysis packages have been developed to generate super-resolution images. To guide researchers on the optimal analytical software for their experiments, in a large community effort we designed a competition to extensively characterise and rank these options [1]. We generated realistic simulated datasets for popular imaging modalities – 2D, astigmatic 3D, biplane 3D, and double helix 3D – and evaluated 36 participant packages against these data. This provides the first broad assessment of 3D single molecule localization microscopy software, provides a holistic view of how the latest 2D and 3D single molecule localization software perform in realistic conditions, and ultimately provides insight into the current limits of the field.

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Superresolution Microscopy Images: What they tell us about protein clusters – and what they don't

The organization and dynamics of proteins and lipids in the plasma membrane, and their role in membrane functionality, have been subject of a long-lasting debate. Developments in superresolution microscopy have facilitated for the first time the direct imaging of cellular structures at length scales far below the optical diffraction limit. Indeed, when applied to the plasma membrane the presence of a variety of protein nanoclusters was revealed, which lead to speculations whether nanoclustering was a general feature of plasma membrane proteins. Particularly in T lymphocytes, clustering of signaling proteins has been proposed to represent a fundamental mechanism for cell activation. Recently, however, doubts were raised whether imaging artifacts inherent to PALM/STORM might have influenced or even caused the observation of some of those protein clusters. To approach these concerns, we developed a method to robustly discriminate clustered from random distributions of molecules detected with single molecule localization microscopy-based techniques like PALM and STORM [1]. In my talk I will present the application of superresolution techniques to different proteins expressed at the T cell plasma membrane. Particularly, I will show that the T cell receptor complex is randomly distributed at the plasma membrane of non-activated T cells [2].

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ILLUMINATING THE SELF-ASSEMBLY OF ALPHA-SYNUCLEIN AMYLOID FIBRIL POLYMORPHS

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Keywords: Correlative SMLM-TEM, PAINT, Polarization, Amyloid fibril polymorphism, self-assembly kinetics

Amyloid fibril formation of alpha-synuclein is a major pathological hallmark of Parkinson's disease (PD). These fibrils exhibit structural polymorphism. There is an increasing evidence implicating different amyloid polymorphs resulting in distinct disease phenotypes [1]. It is therefore imperative to have a detailed mechanistic understanding into the amyloid self-assembly process and structure.

To this end, we performed correlative transmission electron microscopy (TEM) and single molecule localization microscopy (SMLM). We first established an imaging assay that enabled the visualization of the self-assembly process of single amyloid fibrils in real-time. To visualize individual fibrils, we used the PAINT imaging strategy with fluorogenic amyloid binding dyes [2]. We were able to image with unmodified protein monomers and reach high labeling densities on fibrils allowing characterization of respective fibril self-assembly. The unique opportunity of analyzing the polarization of the emitted fluorescence of each binding event enabled us to visualize fibril ultrastructure. To further validate observed structural features, we performed correlative TEM tomography and dynamic PAINT. Such multiparametric correlative imaging allows us to describe fibril growth kinetics with respect to its underlying structure, which would otherwise not be possible.



Figure 1. a) Correlative data of selfassembled fibrils. White arrow indicates the fibril that is analysed in the rest of the figure. b) Localizations are colour encoded according to time (blue to red). Plotting each localization's position along the fibril against time enables the generation of a kymograph to describe single fibril growth kinetics. c) Localizations on a fibril colour encoded according to their respective fluorescence polarization. Plotting each localization's calculated polarization against its respective position along the fibril provides additional information on the underlying fibril structure. d). TEM image of the fibril highlighting its structure.

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Robust and effective adaptive optics in Single-Molecule Localization Microscopy

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Keywords: Adaptive Optics, Deep tissue

Single-Molecule Localization Microscopy has emerged as a powerful microscopy tool to resolve cellular structures with unprecedent resolution. The next challenge is to employ this technique in complex tissues, such as brain slices and organoids. However, here sampleinduced aberrations hamper optimal detection and localization. These aberrations can be overcome by intensity-based adaptive optics (AO) using the single-molecule acquisitions itself, circumventing the need for a guide star or fiducial marker. This is challenging due to the stochastic nature of the single-molecule acquisitions. Several groups have proposed optimization methods to estimate the aberration, but their performance and applicability have remained unclear. To address this, we have systematically compared different optimization strategies using both simulations and experiments. This revealed that none of the proposed techniques achieve robust correction in reasonable signal to noise levels and thus do not guarantee improvement. We next developed a robust method by combining the best features of the existing optimization strategies and then optimized the optimization parameters. We demonstrate that our method can robustly correct initial aberrations of up to 1 rad rms to below 0.3 rad rms (0.9 Strehl-ratio), thus restoring localization precision, in under 300 acquisitions. We used this method to perform dSTORM in hippocampal mouse slices and on microvilli in Caco-cells up to 20 µm deep. Finally, to ensure the widespread application of our approach, we implemented this AO technique as an open source MicroManager plugin that is compatible with a commercially available AO module.

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Template-free 3D particle fusion in localization microscopy

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Keywords: 3D SMLM particle fusion, localization microscopy, super-resolution reconstruction

The achievable resolution in localization microscopy is limited by the localization uncertainty and labelling density. When many identical copies the imaged of structure available are ("particles"), these limiting factors can be eliminated if we can fill in missing information bv fusing information from acquisition



Figure 1 | 3D particle fusion. The result of fusing 50 tetrahedrons using the proposed method (**a**) Top x-y view (**b**) Side view (c) 3D origami design.

of similar structures. Previously, we developed a novel template-free particle fusion method which is capable of aligning 2D particles with very low degree of labelling $(DOL)^1$. As an ongoing work, we have extended our approach to 3D SMLM particles.

The existing methods for 3D particle fusion of SMLM data are either limited to those that use a template² or the ones that copy techniques from cryo-EM³ and fuse 2D projections, not true 3D data. While the former is prone to template bias problem, the latter are ignoring the fundamental differences in image formation of SMLM and EM images, and most importantly cannot handle 3D data acquired typically in SMLM. We developed a method which is template-free and directly works on 3D localization data. It is based on the maximum information that can in principle be extracted from aligning N particles. Firstly, all segmented particles are aligned to each other using a pair registration method. This will provide us with N(N - 1)/2 (redundant) relative motion parameters (3D rotation and x, y, z translation). Then, the Lie-algebraic representation of motion parameters are averaged in the L_1 sense which is subsequently followed by outliers removal and bootstrapping procedures.

We have evaluated our developed method on an experimental dataset containing tetrahedronshaped DNA-origami nanostructures imaged with 3D DNA-PAINT⁴. Figure 1a-b show the result of fusing 50 manually segmented tetrahedrons containing \sim 34k localizations. The reconstruction is fully compatible with the designed 3D patterns of vertices (figure 1c).

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Bioorthogonal labelling of GABA-A receptors for super-resolution microscopy

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Keywords: GABA-A receptor, unnatural amino acids, click-chemistry, dSTORM

Here we incorporate unnatural amino acids in GABA-A receptor alpha 2 subunits to allow click-chemistry labeling providing site specific targeting of receptors for superresolution microscopy.

Superresolution microscopy relies on bona fide labeling strategies using small labels that site specifally react with the respective target protein. Small labels offer low steric hinderance and avoid crosslinking or internalization of surface antigens. Genetic code expansion allows insertion of unnatural amino acids by site directed mutagenesis that can be adressed by tetrazines conjugated to small fluorophores.[1] We show here that GABA-A receptor alpha 2 subunits can be modified by incorporation of unnatural amino acid and can be site-specifically labeled by click-chemistry. In HEK-cells bioorthogonal labeled receptors can be resolved by superresolution microscopy (*d*STORM) and their diffusion can be assessed by fluorescence recovery after photobleaching (FRAP). As a next step we will conduct patch-clamp recordings on HEK-cells expressing clickable GABA-A receptors to verify receptor functionality. Finally, we will conjugate photostable fluorophores to tetrazines to investigate long-term single molecule tracking on basal membranes of COS7 cells in the future.

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Dynamics of the formation of RecA filaments at DNA double strand breaks in Bacillus subtilis

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Keywords: RecA, recombination, repair, DNA, replication fork

In all living organisms, the response to double-strand breaks (DSBs) is critical for the maintenance of chromosome integrity. Homologous recombination (HR), which utilizes a homologous template to prime DNA synthesis and to restore genetic information lost at the DNA break site, is a complex multistep response. In *Bacillus subtilis*, this response can be subdivided into five general acts: (1) recognition of the break site(s) and formation of a repair center (RC), which enables cells to commit to HR; (2) end-processing of the broken end(s) by different avenues to generate a 3'-tailed duplex and RecN-mediated DSB 'coordination'; (3) loading of RecA onto single-stranded DNA at the RC and concomitant DNA strand exchange via RecA; (4) branch migration and resolution, or dissolution, of the recombination intermediates and replication restart in case of stalled forks, followed by (5) disassembly of the recombination apparatus formed at the dynamic RC and continued segregation of sister chromosomes.

When HR is impaired or an intact homologous template is not available, error-prone nonhomologous end-joining directly rejoins the two broken ends by ligation. Single-particle (molecule) tracking (SPT/SMT) is a powerful method to study dynamic processes in living bacterial cells at high spatial and temporal resolution [1].

We have performed single-molecule imaging of early DNA double-strand break repair events during homologous recombination in the model bacterium *Bacillus subtilis* [2,3]. Our data show that presynaptic preparation of DSBs, including loading of RecA onto ssDNA, is highly rapid and dynamic and occurs throughout the chromosome, not only at the replication forks or at distinct sites where many breaks are processed in analogy to eukaryotic DNA repair centres.

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Iterative MINFLUX allows molecular resolution in cells in arbitrarily large regions

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Keywords: MINFLUX, molecular imaging

Superresolution microscopy methods such as STED and PALM/STORM have revolutionized far-field optical fluorescence microscopy by manipulating state transitions of the emitters, offering potentially unlimited resolution. In practice, however, the resolution of an image is limited by the finite photon budget of fluorescent probes, while their finite emission rate imposes a spatial-temporal trade-off in tracking applications. By synergistically combining the strengths of both superresolution families, the recent MINFLUX concept (1) tackles these limitations by rendering each emitted photon more informative.

MINFLUX localizes an emitter by repeatedly probing its location with an excitation beam that features a zero of intensity. The emitter position is obtained from the knowledge of the beam shape and the number of photons collected at each location of the beam. When compared to conventional centroid-localization techniques, it is possible to reach a given precision by using fewer photons, or conversely, have an improved precision for the same photon budget.

In this presentation the iterative MINFLUX methodology will be discussed, which allows the high photon efficiency in arbitrarily large regions. Molecular resolution images in live and fixed cells in two and there dimensions will be presented (see fig. 1).



Fig. 1. A. Micron-sized MINFLUX image of a U-2 OS cell expressing NUP96-SNAP labelled with Alexa Fluor 647 after fixation. Zoomed images show single nuclear pores. B, Histograms of standard deviation of localizations from a single molecule in x and y. C, Evaluation of the localization precision in the image (A). The histogram shows the distance of one localization to the mean position measured from one molecule. **D**. Fourier ring correlation curve for the image in (A). E, Render of insets of (a) with 3sigma ellipses for each molecule. F, MINFLUX image of a living U-2 OS cell expressing NUP96mMaple.

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