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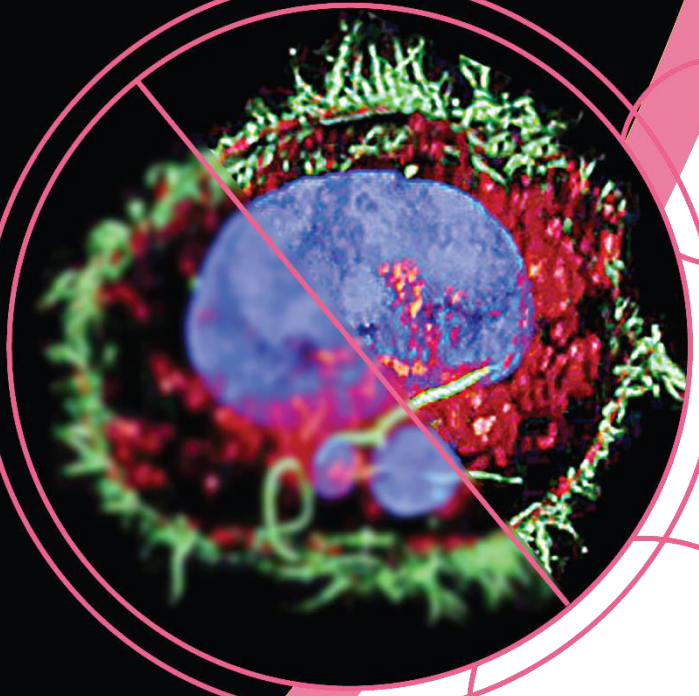

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ABSTRACTS

Keynote Presentation

Time modulated excitation for enhanced single molecule Localization

Abigail Illand[1], Maximilian Lengauer[1,2], Pierre Jouchet[1], Emmanuel Fort [2] & Sandrine Lévêque-Fort [1,*]

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Abstract: In Single Molecule Localization Microscopy (SMLM), the positions of the fluorophores are obtained from a fitted Point Spread Function. This spatially based localization precision will then strongly depend on the PSF shape, which can be degraded by defocusing and aberrations and affect both lateral and axial localization precision, but also the capability to image in depth in complex samples.

We proposed an alternative localization method called ModLoc[1-4] where the uniform excitation is replaced by a time-varying structured illumination over the entire field of view. The illuminated fluorophores have a modulated emission where the phase encodes their position. The demodulation of the fluorescence emission requires extracting four intensities values for each single molecule event, and as emitters can exhibit fast ON-time, we have developed various demodulation strategies based on the introduction of active optical elements that samples the modulated emission in distinct subarrays of the camera to take into account all events. We will present recent developments in particular a new implementation which offers an increased field of view (~50 x 50 μm^2) with a flexible demodulation speed and the capability to reveal multiple dyes based on a specific implementation of spectral demixing. We will in particular present the unique performances of ModLoc to retrieve 3D images of multiple targets at various depths but also in complex samples.

In ModLoc, the structured excitation is generated by a conventional interference pattern, where the periodicity of the pattern requires a camera to unwrap the phase information. To avoid this step and realize a full time coding of the position, we have recently proposed a new strategy, in which we introduced an original dynamic wide field structured excitation which encodes each point in the field of view with a unique modulation frequency. This permits to localize without ambiguity emitters within a large field of view but in combination with monodetectors which offer unique assets in terms of acquisition speed and functional imaging. We will present an example of implementation of TimeLoc and first results.

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Keynote Presentation

In operando single-molecule microscopy

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Abstract: In this talk, I will present an innovative nanofluidic sensing method that utilizes liquid-induced quantum emission from native defects in hexagonal boron nitride (hBN) for molecular imaging. This approach addresses the challenge of directly observing properties of liquids confined at the atomic scale, which traditionally rely on indirect measurements. By activating native defects in hBN with organic solvent molecules, we can observe single-molecule dynamics and gain insights into the local dielectric environment through defect emission spectra. This technique not only enables direct imaging at the molecular level but also reveals increased dielectric order under extreme confinement, showcasing the potential of integrating solid-state nanophotonics with nanofluidics for advanced sensing applications. Furthermore, we explore the development of a nanoscale sensor for optically tracking redox reactions in organic solvents.

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Mayner Eveline, et al. In preparation

Keynote Presentation

pMINFLUX with DNA PAINT and graphene

Fiona Cole[1], Jonas Zähringer[1], Alan Szalai[1, 2], Giovanni Ferrari[1], Lars Richter[1], Jakob Hartmann[1], Merve-Zeynep Kesici[1], Bosong Ji[1], Izabela Kaminska[1], Andres M Vera[1], Tim Schröder[1], Martina Pfeiffer[1], Patrick Schüler[1], Ingrid Tessmer[3], Fernando D. Stefani[2], Philip Tinnefeld[1,*]

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Abstract: Superresolution microscopy has overcome photon-limited resolution with the combination of single-molecule localization and structured illumination e.g. by MINFLUX microscopy [1]. We recently presented pulsed-interleaved MINFLUX (pMINFLUX) that simplifies switching between he excitation donuts and additionally provides fluorescence lifetime information [2]. In this presentation, we will demonstrate the benefits of combining MINFLUX with lifetime imaging for superresolved FRET and for co-tracking of single-molecules in the nanometer range [3]. Combined with graphene energy transfer and DNA PAINT, isotropic resolution below 2 nm is shown on DNA origami model structures [4]. While super-resolution microscopy could robustly provide many coordinates from molecular complexes in real space, resolving multiple coordinates through localization methods requires the system to be

static over the time period of the measurement, typically ranging from many seconds to hours. For superresolution to contribute to dynamic structural biology, the challenge of obtaining robust insights amidst translational or rotational movements undermines certain benefits fluorescence provides for operating at physiologically relevant conditions. To this end, we present Graphene Energy Transfer with vertical Nucleic Acids (GETvNA) [5], a new fluorescence-based method that provides Ångström resolution to study biomolecular complexes involving nucleic acids under biologically relevant conditions. Besides the axial information provided by graphene energy transfer (GET), GETvNA exploits the vertical orientation spontaneously adopted by double-stranded DNA (dsDNA) segments bound to graphene by single-stranded DNA (ssDNA) overhangs. With GETvNA we determine bending angles of distorted DNA structures and visualize protein diffusion with single basepair stepping [5].

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Keynote Presentation

Quantum imaging – From fundamentals to biomedical applications

Sebastian Töpfer[1], Sergio A. Tovar Pèrez[1], Jorge Fuenzalida[1], Marta Gilaberte Basset[2,3], Josué R. León Torres[2,3], and Markus Gräfe[1,2,*]

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Abstract: The exploitation of the quantum properties of non-classical light states has opened up new avenues in imaging, spectroscopy, and sensing [1]. This presentation will focus on quantum imaging that uses correlated and entangled photon pairs. It will cover the basics, including the generation of photon pairs, and provide an overview of the available techniques in this field. This will encompass potential applications, as well as the limitations of quantum-based imaging technologies. The main emphasis of the talk will be on quantum imaging with undetected light [2]. This innovative approach allows for illuminating an object with light that is not detected, while the light that creates the object's image in the camera *has never interacted* with the object. This paradoxical phenomenon demonstrates the unique nature of quantum mechanics and has profound implications for non-invasive imaging techniques.

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Selected Presentation

Estimation of field-dependent aberrations directly from single molecule localization data

Isabel E.A.C. Droste [1], Yutong Wang [1], Erik Schuitema [2], Sjoerd Stallinga [1,*] and Bernd Rieger [1,*]

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Abstract: In SMLM, the location of a fluorescent emitter is determined by fitting a PSF model to the image of a single emitter. A vectorial PSF model that takes into account optical aberrations and emitter dipole orientation can lead to a higher localization accuracy and precision [1,2]. Potentially present aberrations depend on the position in the field-of-view (FOV), but are often taken constant or neglected altogether. The 2D image of a single emitter has too poor signal-to-noise ratio to determine these aberrations. We propose a calibration-free and model-driven approach for fitting field-dependent aberrations directly from single molecule data. Our method links the aberrations of several thousands of emitters across the FOV, strongly reducing the total number of unknowns. We can do this because the variation of the Zernike aberration coefficients over the FOV can be described by low order polynomials according to Nodal Aberration Theory [3]. Subsequently, all emitters are localized with a vectorial PSF model, using the estimated aberrations. We have brought the fitting time for typical datasets to the minute timescale (50.000 fits/sec for 2D data and 20.000 fits/sec for 3D data), by several algorithmic improvements and by implementation on GPU. The algorithmic improvements relate to initial value estimation and a reduction in the required number of Fourier Transforms per iteration step, and will be detailed in the presentation. We have tested our method on 2D and astigmatic 3D localization datasets, and shown that our method can accurately determine field-dependent aberrations and improve the localization accuracy compared to not taking into account aberrations for single molecule data.

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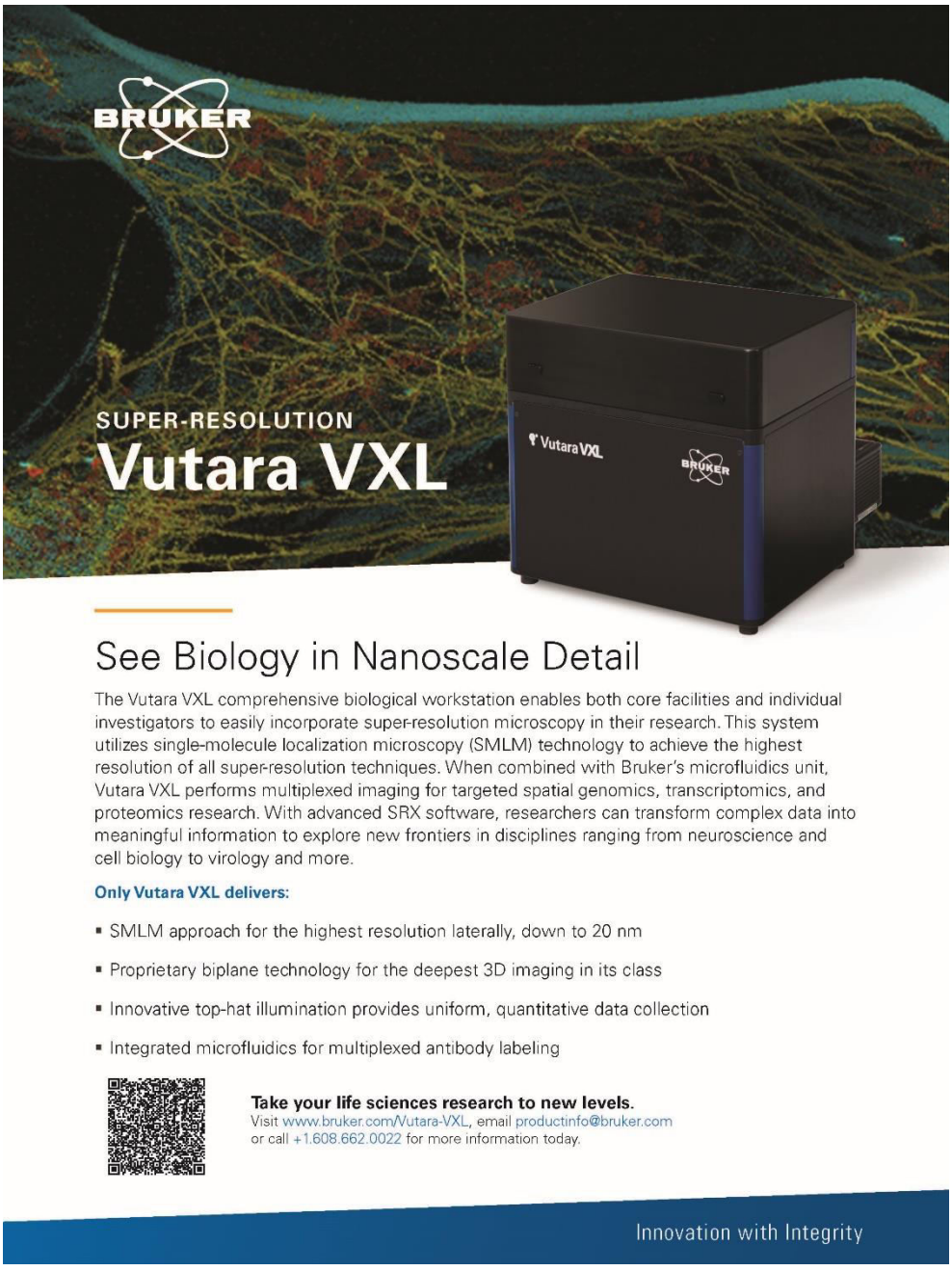
Product presentation

Expanding SMLM to the third dimension with the Bruker Vutara VXL system

Clemens Schneider Bruker Nano GmbH

Abstract: Have you ever considered the possibility of conducting SMLM experiments on biologically relevant structures nestled deep within tissue slices or other thick samples? Perhaps you've encountered limitations due to the restricted penetration depth of your SMLM system or the absence of robust 3D-detection capabilities? Look no further. The Bruker Vutara VXL system is breaking free from the constraints of limited penetration depth and PSF-engineering-based 3D-detection in SMLM. Image wherever in the sample and perform spatially- and probe-multiplexed experiments. Superior system stability allows you to run experiments for serval days, which is crucial under highly multiplexed conditions.



Selected Presentation

Noise amplification and ill-convergence of Richardson-Lucy deconvolution

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Abstract: An important quest in the field of imaging is to devise instruments and methods to deliver the sharpest and most contrast rich images possible. Computational enhancement of raw images acquisitions are an important and broadly applied inroad to do so. This enhancement can be achieved via image processing steps such as filtering operations that are applied ad hoc, agnostic to the underlying physics of the image formation, or via learning-based data driven approaches. Deconvolution, on the other hand, provides an estimate of the underlying object using statistical inference and a model of the image formation. The archetypical algorithm in this field is Richardson-Lucy (RL) deconvolution [1,2]. An important advantage is that it enables reconstruction of out-of-band information, depending on the type of object that is imaged [3].

The application of RL deconvolution to practical imaging settings in astronomy or microscopy has brought to light that the algorithm converges slowly, if at all. Moreover, with increasing number of iteration steps an apparent noise structure builds up, originating from small perturbations of the input due to physical and/or numerical noise. In my presentation, I address the questions why RL deconvolution has a problematic convergence or why the procedure is so sensitive to noise, using a Cramér Rao Lower Bound (CRLB) analysis. As the RL algorithm is a form of Maximum Likelihood Estimation for the ground truth object, a hypothetical well behaved optimum must have a lower bound on the precision of the estimate of the object, and this lower bound is the CRLB. I will show that the CRLB diverges and that hence the original assumption of a regular, well-behaved optimum must be false. It also intimately connects noise sensitivity and noise amplification to a lack of convergence of the iterative procedure. In the presentation I will also provide a review of existing and an outlook on new mitigation strategies for this problematic behaviour.

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Selected Presentation

Image evaluation method to optimize uneven illumination corrections in multimodal microscopy

Elena Corbetta[1,2], Matteo Calvarese[2], Hyeonsoo Bae[2], Chenting Lai[4], David Pertzborn[5], Tobias Meyer-Zedler[1,2], Bernhard Messerschmidt[4], Anna Mühlig[5], Orlando Guntinas-Lichius[5], Micheal Schmitt[1], Juergen Popp[1,2], Thomas Bocklitz[1,2,3]*

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Abstract: Uneven illumination affects all measurements acquired by optical microscopes and strongly impacts large multicolor and nonlinear images, impeding the correct analysis of the measurements.[1,2] Various algorithms for the reduction of uneven illumination exist, but an established workflow for the evaluation of raw and processed images is still lacking.[1-3] To address this issue, we implemented a machine-learning based method to evaluate and enhance (EVEN) images affected by uneven illumination. We trained a Linear Discriminant Analysis model to classify good and bad corrections of uneven illumination in experimental images. The EVEN method was tested on multimodal measurements of human head and neck tissue slices composed of three channels[4]. The trained model was employed to predict the best correction of single channels and to generate an optimized multimodal image. The EVEN method enables the automatic and quantitative assessment of raw and corrected images and the selection of the best processed result. When multiple channels are available, the method can optimize the multimodal measurement by merging the best corrections of single channels. The optimized result simplifies further processing and analysis tasks, such as image registration and segmentation.

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Invited Presentation

Cryogenic Super-Resolution Fluorescence Microscopy

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Abstract: Super-resolution methods present a true game changer for the field of correlative light and electron microscopy (CLEM). They allow bridging the big resolution gap between conventional fluorescence microscopy (FM) and electron microscopy (EM) [1]. Cryo-immobilization by fast-freezing techniques has been introduced to allow imaging in vitreous (glass-like) biological samples with superior structural preservation. On the one side, cryo-EM has evolved into a routine method for structural biology. Particularly cryo electron tomography (cryo-ET) offers insights into intact cells at a resolution down to the Angstrom range [2]. On the other side, super-resolution FM under cryo-conditions is still at a very early and experimental stage [3]. However, the combination of both cryo-microscopy methods has great potential to open up a wide range of new application possibilities in cellular and structural biology. In my talk, I will discuss the challenges, our current solutions and the prospects for super-resolution cryo-CLEM.

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Selected Presentation

Imaging organelles and cellular architecture using new fluorescent probes for live STED and SMLM imaging

Victor Breton[1], Lazare Saladin[2], Paul Nazac[1], Hela Benaissa[3], Thibault Lagache[4], Julie Nguyen[1], Andrei Klymchenko[2], Thierry Galli[1], Jean-Christophe Olivo-Marin[4], Arnaud Gautier[3], Mayeul Collot[2], Lydia Danglot [1*]

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Abstract: Elucidating molecular organization in cell biology requires to precisely localize single or aggregated molecules. In collaboration with A. Gautier, we recently selected new fluorescent chemogenetic reporters (pFAST [3]) consisting in bright synthetic organic dyes anchored to genetically

encoded tiny tag of 14kDa. We will show how this tunable fluorescent chemogenetic reporters can be used in live STED super-resolution microscopy whatever the proteins of interest (Nature Comm 2021 [3]). We previously developed a free plugin SODA (Statistical Object Distance Analysis plugin, Nature Comm 2018 [1]) that uses either micro- or nanoscopy to significantly improve standard co-localization techniques. This method can be used to decipher either contact site in STED [2] or to detect the alignment of synaptic proteins in STORM but need a membrane counterstain.

We previously presented the MemBright family [4] which are six fluorescent probes compatible with long-term live-cell imaging (without any use of transfection or transgenic animals) that can be used in 3D multicolor dSTORM in combination with immunostaining [4]. We are now extending MemBright capabilities to Live STORM imaging that requires probes able to spontaneously blink. Those photoconverters [5,6] can in conventional or SMLM imaging to image lipid droplets, plasma membrane, or mitochondria on live samples (6).

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Product presentation

Nanoscopy: Tailored laser solutions from CW up to femtoseconds

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Selected Presentation

One fits all: A universal laser illumination source that enables new biophysical methods

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Abstract: The flatness of laser-based illumination and the available power have a major impact on image quality and can restrict the contrast and precision of many techniques, including the localisation precision of single-molecule localization microscopy (SMLM). In addition, different laser sets usually need to be provided for different microscope methods, leading to high costs and complicating the development of multimodal devices. We have combined a flat top-hat illumination with a set of high-speed switching lasers and picosecond lasers for fluorescence lifetime applications. Our solution offers a high flatness even at very short µs exposure times, making it compatible with even the fastest cameras and fluorescence microscope techniques. We have proven the applicability of our laser solution for both. wide-field/TIRF and confocal microscopy. It allows to combine multiple methods for simultaneous measurements, such as alternating multi-color excitation schemes, super-resolution and lifetime approaches. Our system supports 5 wavelengths and is more cost-effective than a similar commercially available solution. We were able to implement novel biophysical methods, such as parallelised and camera-controlled 0-mode-waveguide-based single-molecule detection and wide-field fluorescence lifetime imaging recordings.

Selected Presentation

SPAD array detector-based localisations for Tracking and Imaging

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Abstract: The use of a SPAD array detector in a confocal microscope enables to perform Image Scanning Microscopy (ISM) with an improved resolution and signal-to-noise ratio as well as a suppressed out-of-focus signal [1–3]. With small adaptations, the setup enables localisation-based tracking and imaging. For tracking a single emitter, the fluorescence pattern on the array detector (microimage) can directly be used for its localisation. We implemented real time position estimators and recenter the focus to the emitter with galvanometric scanners and an objective piezo. Hence, 3D-Tracking is possible without the use of fast, e.g. electro-optical, beam deflectors. We performed 3D-Tracking with simultaneous lifetime measurement of diffusing beads in water and of lysosomes in living cells [4]. Localisation techniques

using a structured focused excitation, e.g. MINFLUX, can have a larger effective localisation range when combined with an array detector. In addition to the number of detected photons, the microimages are used in position estimation [5]. With ISM-FLUX we measured immobilized gold beads as well as DNA-origami nano rulers with emitter spacings of tens of nanometer. In both setups we verified the single molecule sensitivity of our detectors, which enables in the next steps single molecule tracking and imaging in a confocal microscope.

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Invited Presentation

Advantages of small non-antibody binding proteins (Affimers) in super-resolution imaging

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Abstract: Background: The use of antibodies in super-resolution imaging results in large linkage errors and uncertainty of dye position due to antibody flexibility [1]. Affimers, small non-antibody binding proteins, overcome this challenge [2]. They are small (~12kDa in size, and about 3-4 nm in length) and can be engineered to contain a unique cysteine residue at their N- or C-terminus for specific attachment of a single fluorophore. The Affimer scaffold is based on the plant phytocystatin protein and comprises two variable loops of ~9 amino acids, resulting in a highly diverse library (>10^{10} unique clones)[3]. Affimers specific for a protein of interest are isolated by phage display and then subcloned into bacterial expression vectors for expression, purification and subsequent dye labelling [4,5]. Here we have specifically focused on the advantages of Affimers in a range of super-resolution imaging approaches, including STED [6], dSTORM, DNA PAINT [7] and iPALM. We find that Affimers work well in these approaches, and specifically outperform antibodies in labelling proteins within the Z-disc, a narrow dense (100nm wide) structure found in striated muscles, and we have developed novel analysis approaches to unpick the organization of Z-disc proteins from dSTORM datasets [8,9]. Overall, our data reveals that Affimers are valuable tools that show great promise for super-resolution imaging.

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Invited Presentation

Spectral multiplexing with photoswitchable fluorescent proteins

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Abstract: The observation of organelles dynamics and macromolecular complex interactions inside living cells and tissues requires minimally invasive imaging strategies. In this context, photocontrollable fluorescent proteins (FPs) play a crucial role as tags in optical super-resolution microscopy and functional live cell imaging. To this end we have previously shown that reversibly switchable FPs enable fast (1 Hz for a 50 x 50 μm2) and gentler (< 1 kW/cm2 illuminations) nanoscopy [1]. Additionally, irreversibly photoconvertible FPs can achieve photolabeling with high spatiotemporal precision. Nevertheless, their photophysical complexity poses challenges in expanding such techniques toward multiplexing and in vivo imaging. Here, we explore novel photoswitching mechanisms for fluorescent proteins in the red and near-infrared region of the spectra and assess their compatibility with live cell imaging at the nanoscale (Pennacchietti et al, Nat. Meth, 2018). Finally, we present strategies to combine the spectral and photophysical fingerprint of distinct photocontrollable FPs to achieve multiplexing in live cell imaging at the nanoscale and photolabeling studies (Pennacchietti et al, Nat Comm, 2023).

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Invited Presentation

Investigating cellular logistics with live-cell STED super-resolution microscopy

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Abstract: In the lab we use gene editing and live-cell super-resolution microscopy to investigate the mechanisms of membrane homeostasis in health and disease. To be able to image physiological molecular processes in the crowded cellular cytoplasm in living cells, we developed labelling methods for stimulated emission depletion (STED) super-resolution imaging in living cells [1] and a pipeline for the rapid generation of CRISPR-Cas9 knock ins. These methods unlocked the possibility to image dynamics at sub-50 nm spatial resolution and under near-native cellular conditions. Dynamic nanoscale microscopy of endogenously tagged machinery is revealing novel cellular roles for ARF GTPases, one of the major family of regulators of cellular membrane homeostasis [2]. Defining the role of ARFs led to the discovery of unexplored sorting mechanisms between the endoplasmic reticulum and the Golgi apparatus. We show that bi-directional transport between the ER and the Golgi apparatus occur via a dynamic and quickly remodeling network of connecting nano-tunnels [3]. In a similar way, ARF1 tubular-vesicular compartments serve as a sorting station between Golgi and endo-lysosomal organelles to coordinate short-range secretory and lysosomal trafficking [4].

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Invited Presentation

In vivo STED microscopy of the living mouse brain

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Abstract: Synaptic plasticity lies at the heart of the formation, storage and retrieval of memory, as well as the emergence of cognitive functions in neuronal networks. Synapses, which are connecting nerve cells, are not binary on/off units but undergo changes in synaptic strength by a remodeling within the pre- and postsynaptic elements due to activity. The study of synapses and dendritic spines, tiny neuronal protrusions that mediate most of the synapses has been severely hampered by the diffraction-limit of light microscopy. From all super-resolution microscopy techniques currently available, STED microscopy stands out for its imaging capabilities in tissue and enables the study of sub-cellular structural plasticity and brain function directly in a living mammal.

Now, we advanced the technique of *in vivo* nanoscopy for long-term [1] and multi-label [2] imaging and imaging of the experience-dependent remodeling of the neuronal circuitry [3]. By monitoring individual spines for up to 1 month we show that stable spines undergo strong morphological fluctuations, even under baseline conditions in all their geometrical features comparable in magnitude to activity-dependent processes. By combining spectrally separated excitation and detection with temporal sequential imaging of reversible switchable fluorescent proteins (RSFP) we are able to image multiple components of the synapse at the same time *in vivo*.

Taken together, these advances make *in vivo* STED microscopy a powerful tool for the study of cellular networks in their natural habitat.

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Product presentation

New TauSTED tools for gentle live imaging at remarkable nanoscale

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Abstract: The goal of scientific research is to understand the workings of nature. Given the complex interplay of biomolecules, molecular machines, and higher-order cellular structures, confocal imaging emerged as a fundamental tool owing to the optical sectioning, sensitivity, and the temporal and spatial resolution capabilities. Imaging intricate cellular structures at nanoscale resolution while characterizing the dynamics of multiple species in the context of live specimens are emerging avenues followed to shed light on biological processes. With the advent of STED (Stimulated Emission Depletion), researchers have realized the visualization of intracellular structures at the nanoscale, unveiling insights into cellular behavior, interactions, and function.

In this talk, we will present how STELLARIS STED innovations enable gentle live imaging at the nanoscale. We will show how advances in our TauSTED (1) approach to optical nanoscopy deliver cutting-edge resolution and image quality at low light, key to accessing fast nanoscale dynamics of cellular processes. We will also show how fluorescence lifetime information can be used for multiplex imaging of different markers, keeping the nanoscopic resolution.

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Selected Presentation

Detection and localization of synaptic anomalies in STED microscopy using Deep Learning

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Abstract: Synaptic dysfunction is a key hallmark of neurodegenerative diseases. On a population level, synaptic dysfunction is heterogeneous and tends to worsen with disease progression [1]. We use super-resolution microscopy [2] and deep learning-based anomaly detection to detect nanometric changes in synaptic protein organization at early time points in animal models of neurodegenerative diseases. The nanometric organization of synapses is measured with **St**imulated **E**mission **D**epletion (STED) microscopy in primary neuronal cultures infected with viruses modulating the expression of key proteins involved in disease progression. Unlike traditional quantitative analysis methods, our deep learning approach automatically extracts features, allowing the detection of out-of-distribution synaptic nanostructures, including synthetically created anomalies, perforated synapses, and sparse fluorescent beads. More specifically, we train deep generative models such as normalizing flows [3], variational autoencoders [4] and diffusion models [5] to model the distribution of anomaly-free, nominal samples such that the networks can highlight regions deviating from the nominal distribution at inference time. Moreover, we adapt the Multiple Instance Learning framework to efficiently handle large images and uncover small and rare anomalies concealed in large fields of view. In ongoing work, the proposed methods are being applied to disease models of neurodegeneration and could lead to discoveries of rare synaptic events that would be overlooked by classical detection and classification approaches of known features, thus radically changing the study of neurodegeneration.

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Invited Presentation

Quantitative bioimaging across scales

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Abstract: New perspectives of mechanobiology are emerging in the biomedical sciences. Recent evidence indicates that immune cells regulate their cell mechanics not only downstream of signalling events triggered by ligand–receptor binding, but that cells employ a diversity of feedback mechanisms to dynamically adjust their mechanics in response to external stimuli. Quantifying cellular forces has therefore become an contentious challenge across multiple disciplines at the interface of biophysics and immunology. Mechanical forces are especially important for the activation of immune T cells. Applying sensitive quantitative super-resolution imaging and force probing methodologies to analyse resting and activated T cells, we demonstrate that the kinetics of the antigen engaging the T-cell receptor controls the nanoscale actin organisation and mechanics of the IS. Using an engineered T-cell system expressing a specific T-cell receptor and stimulated by a range of antigens, force measurements revealed that the peak force experienced by the T- cell receptor during activation was independent of the kinetics of the stimulating antigen. Conversely, quantification of the actin retrograde flow velocity at the IS revealed a striking dependence on the antigen kinetics. Novel ultra-thin superextended lightsheet technology allowed to correlate early calcium activation signalling, IS formation, and effector function. Taken together, these findings suggest that the dynamics of the actin cytoskeleton actively adjusted to normalise the force experienced by the T-cell receptor in antigen specific manner. Consequently, tuning actin dynamics in response to antigen kinetics may thus be a mechanism that allows T cells to adjust the length- and time-scale of T-cell receptor signalling. In the future, the Biophysical Immunology Laboratory aims to translate the established technologies from single cells to live tissues in the Oxford-ZEISS Centre of Excellence.

Invited Presentation

High-fidelity 3D live-cell nanoscopy through data-driven enhanced super-resolution radial fluctuation

Romain F. Laine [1], Hannah S. Heil [2], Simao Coelho [2], Jonathon Nixon-Abell [3], Angélique Jimenez [4], Theresa Wiesner [4], Damián Martínez [2],Tommaso Galgani [5], Louise Regnier [5], Aki Stubb [6], Gautier Follain [6], Samantha Webster [7], Jesse Goyette [7], Aurelien Dauphin [8], Audrey Salles [9], Siân Culley [1], Guillaume Jacquemet [6], Bassam Hajj [5,*], Christophe Leterrier [4,*], Ricardo Henriques [2,*]

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Abstract: Image reconstruction based on fluctuations proves exceptionally powerful for long-term live-cell imaging, surpassing resolution limitations by extracting super-resolution details from brief image sequences captured at low light levels [1]. The latest iteration, eSRRF (enhanced-SRRF) [2], not only enhances reconstruction accuracy but also integrates automated parameter optimization based on image fidelity and resolution. Based on this quantitative image quality metrics a reconstruction parameter combination yielding the best compromise between both measures is automatically suggested to the user. This feature helps to maximize image resolution, while circumventing reconstruction artifacts [3] and minimizing user bias. As an image processing approach eSRRF can be applied to a wide range of microscopy approaches for 2D superresolution imaging. To go beyond this, achieving high-fidelity 3D live-cell nanoscopy, we have also extended the eSRRF reconstruction algorithm to 3D. Realizing 3D eSRRF requires the simultaneous detection of fluorescence fluctuations across multiple focal planes, facilitated by a multifocus microscope (MFM) [4]. This innovative approach has enabled volumetric super-resolution imaging in live cells, boasting acquisition speeds of approximately 1 volume per second. Notably, it has enabled the super-resolution visualization of the dynamic rearrangement of the mitochondrial network in U2OS cells in 3D, capturing changes over several minutes.

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Selected Presentation

Challenges and biophysical applications of MINFLUX Single Particle Tracking

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Abstract: Single Particle Tracking (SPT) is one of the most applied techniques in Biophysics to study the dynamics of individual biomolecules at work in living systems. Conventionally, SPT measurements have taken place with various, mostly diffraction limited, microscopy techniques, with contrast provided by fluorescence, such as in the case of TIRF microscopy, or through scattering, such as widefield or interferometric scattering microscopy. While they are capable of delivering significant results, these methods do however present some disadvantages in their applications, namely the need of large scatterers for scattering-based microscopy, or limited number of localization and framerate for fluorescence-based detection. In this landscape, the introduction of MINFLUX microscopy offers a new groundbreaking tool for SPT. In fact, the nature of MINFLUX detection allows to collect a large number of localizations for each molecular trajectory, while obtaining framerates in the order of tens of kilohertz. Furthermore, the possibility of direct 3D tracking opens new avenues for SPT that could previously not be fully explored.

In this talk, we will explore some of the features and application of MINFLUX-based SPT, originating from experience with the commercial implementation of MINFLUX microscopy, which is based on the iterative scanning approach [1]. First of all, a perspective will be given on the range of applicability of MINFLUX SPT. As several position have to be scanned around a particle to estimate its position, not all particles can be effectively tracked, with respect to their diffusion rate. Secondly, a few applications of MINFLUX SPT, in 2D and 3D, will be showcased, to demonstrate the potential that this technique has to offer to explore the dynamics of single biomolecules in live cells.

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Product presentation

All in One – Teledyne Scientific Imaging: Perfect solutions for Single Molecule, Super Resolution, Light Sheet, Quantum Imaging and lots more

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Abstract: The goal of scientific research is to understand the workings of nature. Given the complex interplay of biomolecules, molecular machines, and higher-order cellular structures, confocal imaging emerged as a fundamental tool owing to the optical sectioning, sensitivity, and the temporal and spatial resolution capabilities. Imaging intricate cellular structures at nanoscale resolution while characterizing the dynamics of multiple species in the context of live specimens are emerging avenues followed to shed light on biological processes. With the advent of STED (Stimulated Emission Depletion), researchers have realized the visualization of intracellular structures at the nanoscale, unveiling insights into cellular behavior, interactions, and function.

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Selected Presentation

Challenges and biophysical applications of MINFLUX Single Particle Tracking

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Abstract: Peroxisomes are small (100-300 nm) intracellular organelles that fulfil many anabolic and catabolic functions. Mistargeting of peroxisomal proteins, which are imported from the cytosol, therefore leads to severe diseases making this organelle an object of research of utmost importance for medical applications. The import of proteins is mediated in a by now unknown manner through a

transient translocation pore which assembles either in a way similar to pore forming toxins or through a liquid/liquid phase separated matrix. We here aim at adding knowledge about peroxisomes using a toolbox of advanced (super-resolution) microscopy methods [1]. We use STED and Minflux super-resolution microscopy and spectroscopy to study the distribution of peroxisomal proteins involved in protein translocation on the peroxisomal membrane as well as we studied the diffusion dynamics of the peroxisomal import receptor and its cargo proteins in the cytosol of cells [2]. Further, we employ a photoactivatable protein to induce and quantify peroxisomal import as well as we study peroxisomal movements in living cells through fast time-lapse imaging and sophisticated image analysis [3].

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Selected Presentation

Automated and event-triggered targeted MINFLUX microscopy

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Abstract: MINFLUX [1] microscopy has recently emerged as a powerful microscopy technique, both for imaging and single-molecule tracking, achieving single nanometer spatial resolution and rapid microsecond temporal resolution. However, recording times for imaging are often hours, and tracking can be challenging to correlate to other cellular processes, potentially limiting the application areas. Here we present automated control of MINFLUX microscopy acquisitions that rapidly targets MINFLUX tracking and imaging to small cellular regions of interest: event-triggered MINFLUX (etMINFLUX). A Python-based open-source software widget, based on that of event-triggered STED [2], is developed to control MINFLUX acquisitions on a commercial Abberior microscope. The widget runs real-time image analysis on confocal images, detects regions and events of interest, and immediately targets MINFLUX acquisitions at those sites. It can further follow targeted regions over time, ensuring MINFLUX is always performed at the dynamic point of interest and correlating the data over the two modalities. Overall, this allows experiments not feasible by manual control as well as more efficient data acquisition. We apply etMINFLUX to membrane-lipid tracking at caveolae sites and virus budding sites in living cells, instantaneously and over a time scale of minutes as the sites evolve, allowing rapid and correlated investigation of local nanometer scale membrane compartmentalization and properties with unprecedented resolution.

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Selected Presentation

Investigating the geometry of 3D molecular movement with MINFLUX microscopy enabled Single Particle Tracking

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Abstract: The recent introduction of MINFLUX microscopy has opened the gates for reliable, high spatio-temporal resolution, single particle tracking experiments in 3D, delivering thousands of continuous localizations with ~10nm dynamic localization precision. Therefore, the instrument is especially suited to measure single molecule diffusion in three dimensions, achieved by continuously updating the localization estimate.

In treating localization data as dense point clouds, we can construct geometrical and morphological features, that can in turn be used to quantize diffusive behavior within and across particle trajectories. We explore the possibilities of this approach on MINFLUX tracking data of *HALO*-tagged SRC protein diffusing on the inner leaflet of model neuronal cells and showcase diffusion mode separation and low-level parameter extraction. By evaluating the paths taken by particles through our topological approach, we also reconstruct raw partial three-dimensional representations of complex samples that allow for surveying geographical parameters. Through shifting the focus to sub-trajectory structures, we unravel highly localized particle behavior at the nanoscale.

With this model-free, data-led approach we aim to complement model-bound diffusion parameter extraction methods [1], enabling detail-on-demand probing of molecular diffusion.

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Invited Presentation

The power of SIM to study the fundamental aspects of chromatin biology

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Abstract: Interference-based linear structured illumination microscopy (SIM) is a powerful approach for multi-color volumetric super-resolution imaging of single cells [1], e.g. to obtain contextual information of dense polymer structures, such as chromatin organization in mammalian cell nuclei [2]. In my talk, I will present our recent work on the biological application of quantitative SIM to study the dynamics of Xist-RNA spreading during X-chromosome inactivation [3] and investigate cell-cycle-dependent cohesion complex organization relevant for sister chromatid cohesion and loop extrusion [4]. In both cases, we utilized the hitherto unrecognized potential of SIM to resolve single molecules/complexes and to quantify their stoichiometry, kinetics, and interaction behaviors *in situ* and in specific cellular states. In both case studies, we made unexpected discoveries that add to our understanding of two fundamental biological processes, highlighting the power of SIM as an unmatched tool for discovery.

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Selected Presentation

Multi-angle TIRF and 2D super-resolution structured illumination microscopy with up to 20 nm axial resolution and 230 µm field of view

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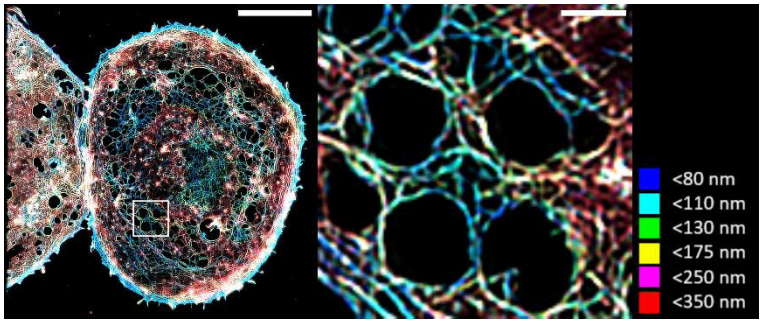
Abstract: Super-resolved structured illumination microscopy (SR-SIM) is among the most versatile and least perturbing fluorescence microscopy techniques capable of surpassing the optical diffraction limit and providing a high spatio-temporal resolution. We have developed a fiber-based structured illumination microscope that overcomes limitations of the field of view and axial resolution without sacrificing speed, image quality or multi-color capability [1,2]. By combining multi-angle (MA) TIRF illumination with SR-SIM, axial resolutions down to 20 nm and lateral resolutions of less than 100 nm are achieved. The FOV is up to 230 µm and is just limited by the high NA objective lens. This is implemented by custom-built, novel components such as a fiber switch, a hexagonal holder and a prism telescope, allowing to seamlessly and quickly manipulate illumination pattern spacings to provide 2D-, TIRF- and MA-TIRF-SIM illumination. The pattern transition times are 1 ms, the SIM frame rate is camera limited at 44 Hz. The maximum FOV is 230 µm in diameter with a 40x 1.4 NA objective lens or 100 µm with a 60x 1.5 NA lens without aberrations or reconstruction artefacts and a resolution between 90 nm and 120 nm for green emission. A modulation depth of 90% on the entire FOV is achieved. Multi-color MA-TIRF-SIM and 2D-SIM is applied on isolated livers sinusoidal endothelial cells and thin tissue sections. Both methods are live-cell compatible, due to low irradiation powers and low raw frame numbers required for a super-resolved image reconstruction.



Multi-angle TIRF-SIM image of a liver sinusoidal endiothelial cell revealing the ultrastructure of the actin cytosceleton with 100 nm lateral and down to 20 nm axial resolution. Scale bar is 10 µm and 1 µm (inset).

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Product presentation

Structuring with a twist: how a Lattice pattern can improve temporal and spatial resolution in wide-field imaging

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Abstract: Structured Illumination Microscopy (SIM) has become the favorite super-resolution technology for live cell imaging. We will present improvements on the encoding and decoding parts of SIM that opened the possibility to distribute the photon budget according to the need of the experiment. The employment of a 2D lattice grid enables to reduce the numbers of required raw images thus adapting the system perfectly to the special needs of capturing fast dynamics in living cells. In addition, sample-dependent resolution well below 100 nm can be achieved.

Invited Presentation

Single objective light sheet allows volumetric super-resolution imaging of thick samples

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Abstract: Single-objective light sheet (commonly named HILO) [1] is a widely implied optical configuration which drastically reduces background fluorescence by selectively illuminating a confined portion inside the sample. We set a simple model ,based on Gaussian optics theory, that predicts the inclined beam features, such as the thickness and the confocal parameter, at the sample level and we validated it through near- and far-field measurements [2]. Notably we further confined the illumination through a linear slit which restricts the thickness of the light sheet below 3 μm, while retaining a FOV of 10 x 40 μm^2. When applied to STORM/PALM imaging, such a confinement does not impact significantly on the image resolution, while it dramatically increases the number of localizations, thus decreasing the time required for images acquisition. Furthermore, we show how to achieve volumetric PALM of thick samples through scanning of the ultra-confined light sheet along the optical axis of the objective. In fact, we achieved single molecule localization of endogenous Efflux Pumps (EPs), fused with PAmCherry, in bacterial biofilms and we quantified EPs' expression levels under native regulation for the first time over the biofilm volume, revealing a decrease in pumps density with increasing depth inside the biofilm and over its maturation course.

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Selected Presentation

Correlative super-resolution fluorescence imaging of liver sinusoidal endothelial cells on transparent polymer photonic waveguide chips

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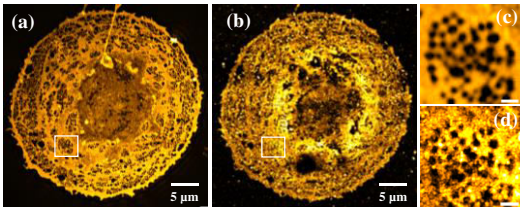
Abstract: Super-resolution optical microscopy (SRM), such as structured illumination microscopy (SIM), stimulated emission depletion microscopy (STED), single-molecule localization microscopy (SMLM) etc., have shown immense potential to visualize the subcellular structures of biological samples beyond the diffraction limit of light [1]. However, the practical implementation of each nanoscopy techniques is still limited in routine biological settings due to multiple reasons such as low throughput, bulky, high cost, and complex systems etc. For example, SIM can acquire images at video rate with low phototoxicity but resolution improvement is limited to 2x [2]. On the other hand, SMLM, e.g., direct stochastic optical reconstruction microscopy (*d*STORM) provides higher resolution, on the order of tens of nanometers, but requires the time consuming acquisition of thousands of raw images [3]. In order to utilize the specific strengths of each technique a combined approach in the form of correlative SRM is important. This study presents the correlative SRM imaging of the intracellular microstructures of liver sinusoidal endothelial cells (LSECs) with SIM and *d*STORM techniques facilitated by a transparent polymer photonic waveguide chip [4]. LSECs contain a large number of pores in their plasma membrane, known as fenestrations with a diameter of 50 – 300 nm. The shape, diameter and number of fenestrations are altered during liver disease, old age, and drug interactions etc. [5]. Hence, the detailed identification of this ultrastructural morphology is important to analyze the cells' functionality. Correlative SIM and *d*STORM microscopy combines high throughput with large fields of view (~120 µm × 200 µm) with high resolution (~ 30 nm). Furthermore, the waveguide chip based TIRF excitation yields a significant reduction of background signals. This study suggests that live cell imaging and study of the dynamics of fenestrations in LSECs on a large scale should be possible.



Correlative 3D-SIM (a, c) and waveguide TIRF based dSTORM (b, d) images of fenestrations in a rat liver sinusoidal endothelial cell (LSEC). Scale bar (a, b) 5 µm ; (c, d) 500 nm.

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Selected Presentation

Applying the visual programming tool JIPipe to quantify super-resolution microscopy images of bacterial surface proteins

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Abstract: When it comes to biological image analysis, the community standard of choice has been ImageJ. However, this stalwart lacked a user-centric visual programming language (VPL) that supports expeditious workflow development at all levels of complexity based on the FAIR (Findability, Accessibility, Interoperability, and Reusability) principles. Accordingly, we developed a new VPL, termed Java Image Processing Pipeline (JIPipe, www.JIPipe.org), which provides nearly 2000 functionalities of ImageJ and its extensions in a fully visual format. Applying JIPipe assures the full reproducibility of the algorithmic procedure, creates a standardized results format, scales automatically towards batch analysis, applies parallel processing with GPU support, and integrates machine learning tools with support for Python and R scripting. JIPipe has also been applied and extended to support the quantification of images provided by the increasingly available super-resolution techniques. Here we use JIPipe to characterize the dynamic membrane localization of bacterial surface proteins in *Streptococcus pneumoniae using 3D microscopy images acquired via SIM and SIM2 microscopy. As* a gram-positive human pathogen, *S. pneumoniae* can evade the host innate immune system aided by several proteins with diverse functions (transport channels, carbohydrate metabolic enzymes, iron receptors) that protrude to the extracellular space. We were interested in observing the subcellular localization of a group of these proteins and peptides located at the cell wall, during an immunological challenge. The JIPipe-based reconstruction of the protein-specific signals over the bacterial volume indicated that the protein distribution was not homogenous along the bacterial surface, but rather concentrated along an equatorial plane of the bacterium. Average distances to the nearest neighbors showed a similar, equatorial distribution, and revealed a linear relationship between the fluorescence intensity of the clusters and the average distance to their nearest neighbors. End-point experiments of peptide distributions using various strains and growth conditions were examined using the JIPipe-implementation of template matching algorithms to reveal the various stages of development during cell division. Our findings point to a protective effect with a dynamic and fine-tuned allocation of bacterial proteins to the cell wall to avoid areas with simultaneously high protein density and intensity. This novel view combined with the optimized analytical tool JIPipe will provide further insights into the dynamic spatial positioning of pneumococcal proteins, thus opening new ways to reassess how pathogens exploit their protein machineries to evade the immune system.

Invited Presentation

From centimeters to nanometers: Novel tools for Quantitative Multi-Modal Imaging

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Abstract: Neurological disorders have a tremendous and often devastating health and psychological impact on individuals, families, and society. Early symptoms of many neurodegenerative diseases can be subtle and nonspecific, making them difficult to identify in the prodromal stages. Their early detection and diagnosis, however, is crucial for effective interventions to prevent rapid disease progression and promote better health outcomes, including improved survival rates, reduced complications and disability, enhanced quality of life, and decreased treatment costs. However, current methods for early detection and diagnosis of neurological diseases have limited accuracy, specificity, and reliability. For example, many diagnostic criteria for neurodegenerative diseases are based on subjective cognitive and behavioral assessments, such as cognitive/memory tests and rating scales, which can be influenced by external factors such as patient mood and education level. Despite significant progress in understanding the pathophysiology of different neurodegenerative syndromes and their genetic risk factors, the molecular mechanisms that drive the onset of these diseases often remain unclear. Moreover, the lack of specific and sensitive (molecular) biomarkers for these diseases makes it challenging to accurately diagnose early stages.

Hereditary spastic paraplegia (HSP), for instance, is a prototype of a genetically very diverse group of neurological disorders that share the degeneration of axons of cortical motoneurons thus leading to progressive spasticity, weakness, and muscle atrophy. Although HSP usually mainly affects lower limbs, it is often complicated by other symptoms such as sensory deficits, incontinence, parkinsonism, cognitive decline to name a few. So far, genetic testing resolves only roughly half of the cases and can then also help to identify persons at risk. The other half, however, remains unclear. Moreover, genetic testing usually does not allow a reliable prediction for disease onset, its progression or the outcome of the disease.

For many neurological disorders, subtle changes in posture, facial geometry and symmetry, and cognitive ability, i.e. macroscopic phenotypes, coincide with progressive alterations in the molecular machinery of cells and the nanoscale geometry and organization of sub-cellular organelles and proteins. For example, neurons are highly specialized cells with specific requirements for structural and functional subdomains that maintain polarity, extend and repair their axonal and dendritic protrusions, and organize sites for synaptic transmission, which can already be distorted at early onsets of neurodegenerative disorders. Distortions involve all parts of the cell from the plasma membrane over the cytoskeleton to intracellular organelles such as the endoplasmic reticulum (ER) and the endo-lysosomal/vesicular system. Consequently, a thorough quantitative, holistic, and multiscale analysis is needed to precisely identify and interpret identities and causalities of such disorders.

Here we present our latest efforts to combine nanoscale super-resolution imaging with structured light stereophotogrammetry to investigate neurological disorders across 9 orders of spatial scale in the future.

Selected Presentation

Nanotexture enables computational multiplexing of super-resolved intracellular structures with high fidelity

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Abstract: We introduce a set of novel multiplexing approaches using organelle-specific nanotextures called NanTex for monochromatic, super-resolved image data. Firstly, we demonstrate ad-hoc multiplexing of spectrally identical labeled organelles in single SMLM images based on nanoscale Haralick-feature [1] extraction. Next, we introduce AI-enabled textural demixing, AI-NanTex with supervised Unet [2] feature generation, trained on publicly available (Shareloc [3]) and dedicated experimental single-organelle SMLM data. AI-NanTex enables the regressive extraction of multiple complex structures (e.g., microtubules, clathrin, endosomes, ER, and actin) from single-channel grayscale SMLM images. This context-agnostic texture recognition relies on probabilistic demixing rather than classical image segmentation, accurately identifying multiple organelles even when heavily overlapped. Furthermore, our method is readily applicable to monochromatic, multi-organelle MINFLUX [4] data without requiring additional training, showcasing the broad relevance of the nanotextural concept across different super-resolution imaging modalities. Texture-sensitive nanoscopy expands the possibilities of multi-color imaging by enabling straightforward multiplexing of the best-performing dyes in complex biological contexts.

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Selected Presentation

Interpretability of Deep Learning Models for microscopy image analysis

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Abstract: Deep Learning (DL) models can be appealing for quantitative analysis in microscopy images [1] but often lack interpretability to provide users with the relevant parts of an image leading to a decision. Recently, we introduced MICRA-Net [2], a DL model that is trained from a simple classification task, from which we extract interpretable information [3] that is used to solve more complex tasks, such as semantic segmentation and detection. For instance, MICRA-Net generates a more detailed semantic segmentation of neuronal F-actin nanostructures in STED images compared to weakly-supervised DL baselines. MICRA-Net was also used to assist experts in locating sparsely labeled axons from electron microscopy images, resulting in 25% more detections compared to manual annotation.

When few annotations are available, Self-Supervised Learning (SSL) can be used to extract rich and robust representations by leveraging large unlabeled datasets [4]. In preliminary results, we demonstrate that learning to differentiate between neuronal proteins in STED images from SSL features learned on a large dataset of out of domain images or a small microscopy dataset results in similar performance. When integrating interpretability approaches [5] we observe that learned SSL representations from microscopy images are better adapted at extracting biologically relevant information. This highlights the need to integrate interpretability methods within the analysis pipelines to ensure the relevance of the learned features by DL models.

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WORKSHOPS

Workshop

Deep Learning for Optical Nanoscopy: Addressing Challenges, Avoiding Pitfalls, and Maximizing Applications

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Abstract: This workshop aims to explore the use of Deep Learning (DL) techniques or pipelines in the context of super-resolution microscopy. Implementing DL frameworks in super-resolution microscopy comes with its own set of challenges and pitfalls. We will go through the steps and precautions that researchers should take when leveraging DL pipelines for their own research.

The common challenges that are faced when applying DL techniques in super-resolution microscopy which includes : 1) the scarcity of annotated data, 2) the difficulty in defining ground truth annotations, 3) the challenges associated with the evaluation of DL models, 4) the black-box nature of DL models, and 5) the need for interpretability will be discussed. Different strategies that can be leveraged to face these challenges will be explored, such as data augmentation techniques to address limited annotated data, model interpretability methods, and transfer learning approaches to improve model generalizability across different microscopy modalities or experiments. Tools to account for and detect batch effects which can be detrimental in analysis pipelines will also be addressed.

By the end of the workshop, attendees will gain a small overview of the current landscape of DL in super-resolution microscopy and will be equipped with practical strategies to address the challenges, avoid pitfalls, and maximize their use of DL models for the quantitative analysis of optical nanoscopy images.

Workshop

Smart microscopy for everyone

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Abstract: Microscopes today produce more than just an enlarged image. They are often part of a complex biological protocol, where, for example, various staining steps in combination with structured illumination (e.g. SIM) microscopy lead to an improved resolution of the image.

ImSwitch [1], a Python-based software that revolves around the multidimensional image viewer "napari" allowing the orchestration of different hardware modules like cameras, focusing stages, and lasers, enables the realization of smart microscopy setups. Here, an experiment cannot merely be handled step by step in the traditional sense, but rather dynamically, event-based. The microscopy image can be analyzed in real-time and then adaptively deliver a control command to the hardware. ImSwitch is additionally equipped with a variety of external interfaces, such as the REST API, so that it can be integrated into existing workflows, where, for instance, a pipetting robot prepares the sample, and a robotic arm moves the sample to the microscope before it scans the sample in a high-throughput process. Within the workshop, we want to demonstrate the range of functions of ImSwitch using various examples of open-source microscopy hardware. For this purpose, we have a UC2-based light-sheet microscope [2] that allows the fast capture of 3D volumes, as well as a fully automated XYZ microscope for the examination of histological samples. An open-source and fully 3D printed setup shows how iSCAT can be brought closer to a wide audience. The device is operated by the also open-source ESP32-based microscopy controller, so that a feedback-loop oriented online data processing within ImSwitch allows for an instantaneous data output.

The workshop is intended to show users how ImSwitch represents an entry into the world of smart microscopy and how it can be adapted for one's own system. The open-source core already includes a variety of device drivers and can be expanded, for example, by those from MicroManager. For further information on that matter have a look in our online documentation [3].

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POSTERS

F-Actin fluorescence staining evaluated using 2D polarization fluorescence imaging (2DPOLIM), structured illumination microscopy (SIM) and fluorescence lifetime imaging (FLIM)

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Abstract: Actin assembly and disassembly is essential for any type of cell mobility. Variations in Actin content in liver tissue have been found to be an indicator for animal survival in a recent study utilizing a mouse model for systemic infection.[1] In vivo imaging of Actin, thus, provides access to enhanced understanding of cellular behavior in various areas of research including the response to infection and therapy. We evaluate approaches for in vivo Actin staining by comparing results obtained using 2DPOLIM, structured illumination and fluorescence lifetime imaging (FLIM).

2D polarization fluorescence imaging (2D POLIM) was developed at Lund university.[2] By combining a controlled variation of the orientation angles of linearly polarized excitation and fluorescence detection, it provides complete in-plane information of the polarization state of the sample. 2D POLIM was successfully applied to a semi-quantitative investigation of early protein aggregation of GFP-labeled human α-synuclein in models of Parkinson's disease ex vivo.[2] In a recent study, we applied 2D POLIM to phalloidin-DY490 stained liver tissue of mice from different treatment groups in the context of polymicrobial sepsis. Qualitative analysis revealed significant differences in the molecular arrangement of F-actin. Furthermore, the observation agrees with the survival for the animals in the different treatment groups.[1,3] These findings from genetically encoded and stained tissue are promising for designing further studies using 2D POLIM to enhance the understanding of pathways in infectious diseases.

2DPOLIM provides information on the average state of F-Actin aggregation within the diffraction limit. Therefore, the results obtained using 2DPOLIM are compared to results obtained using structured illumination microscopy (SIM) and fluorescence lifetime imaging (FLIM).

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Detecting nanoscopic changes to mitochondrial morphology in human models of Parkinson's Disease

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Abstract: Mitochondria are dynamic and sensitive organelles responsible for providing the majority of the brain's energy [1], and their dysfunction has been implicated in Parkinson's Disease (PD) [2]. In this project, we aim to show that PD alters mitochondrial morphology at the nanoscale.

To identify how PD affects mitochondria in humans, we characterized mitochondrial morphology features in dopaminergic neurons derived from PD patient stem cells [3] harbouring genetic mutations and compared them to controls. By comparing images taken with confocal microscopy and STimulated Emission Depletion (STED) microscopy [4], we showed that visualizing mitochondria at the nanoscale with super-resolution tools allows for precise characterization of mitochondrial morphology and aids in describing how this morphology is altered in PD.

Preliminary results show that confocal images cluster multiple closely packed mitochondria as single objects and therefore artificially increase average mitochondrial size. When these same images were examined with STED microscopy, we found that mitochondria in PD-associated neurons have a more fragmented phenotype than those in controls. The PD-associated mitochondria tend to have smaller areas, smaller aspect ratios, and increased circularity. Future work includes investigating the effects of PD on mitochondrial morphology in different neuronal compartments by comparing somatic, dendritic, and axonal mitochondria between the control and PD groups.

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Measuring lipid dynamics and packing with MINFLUX microscopy

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Abstract: The eukaryotic cell plasma membrane is highly complex and its organization is critical to multiple biological processes, such as virus infection. Still, the role of membrane heterogeneity remains poorly understood due to the lack of techniques showing both high spatial and temporal resolution. Here, we assess the capacity of MINFLUX (minimal photon fluxes) microscopy to investigate lipid membrane organization quantitatively. We show that MINFLUX imaging combined with exchangeable solvatochromic dyes allows quantifying lipid packing in live cell plasma membranes with nanometre resolution. Further, we use MINFLUX in combination with fluorescent lipid analogues to follow their path over space and time. We explore the time resolution of such single lipid tracking experiments as well as apply it to investigate Gag-induced lipid sorting on HIV virus assembly sites at the plasma membrane. To separate lipids diffusing in the plasma membrane from those located in a virus budding site, we develop an open-source MINFLUX track segmentation and analysis pipeline based on a confocal fluorescent image reference. In conclusion, we highlight the applicability of MINFLUX imaging and tracking to investigate lipid organization and dynamics in live cell membranes.

Dynamic single particle measurements with an high throughput iSCAT-TIRF setup

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Abstract: Interferometric Scattering Microscopy (iSCAT) enables label free, prolonged and high frame rate image acquisition. Typically, iSCAT setups employ scanning illumination schemes to achieve uniform sample illumination. However, this implementation limits the field of view (FoV) and maximum sampling rate, while increasing hardware requirements and setup size. We demonstrate that it is possible to achieve a large (60µm x 60µm) uniformly illuminated FoV through a passive refractive optical element in the iSCAT illumination path and record images with a time resolution of 300 µs. Thus, we developed a scanning-free Interferometric Scattering Microscopy setup in combination with an objective based Total Internal Reflection Microscopy (TIRF) channel. This setup has the additional advantage of a compact footprint (1m x 1m) and a freely available software implementation (ImSwitch). We show that we can acquire images with a uniform contrast and a <10 nm localization precision throughout the FoV and track single particles with a dynamic localization precision <20 nm for prolonged time thanks to a focus lock channel. The instrument can perform quantitative single molecule measurements as Single Particle Tracking and imaging Fluorescence Correlation Spectroscopy. We demonstrate and compare these measurements on different model membrane systems. This illumination-detection scheme for iSCAT has the potential to drive further innovation and applications of iSCAT microscopy in the life Sciences.

Studying the distribution of peroxisomal proteins with STORM and Minflux

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Abstract: The peroxisome is known to be a small membrane-enclosed metabolic organelle that encapsulates enzymes and their substrates. The enzymes, that constitute the peroxisomal matrix, are responsible for multiple physiological functions, therefore a defect in import of the peroxisomal proteins leads to different pathologies. In addition, the peroxisomes can modify their shape, size and number in response to metabolic needs or environmental changes. Hence, it becomes apparent that not only the volume of peroxisomes will be affected by these dynamics, but also a change in shape and size can affect the organelle function[1]. Therefore, we are applying super-resolution microscopy techniques, such as STORM and Minflux, to study the morphological differences of peroxisomes and the distribution of peroxisomal proteins in the organelle. With these methods, we could already get an impression of the morphology of peroxisomes, which will be further analyzed with cluster analysis like DBSCAN. The goal is, on one hand, to test different labeling strategies for the peroxisomal proteins and on the other hand, to compare the morphological features of peroxisomes with different super resolution techniques. Here the peroxisome is a great test object to see if their morphological features can be resolved with different labeling and imaging strategies.

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Integral field Raman spectroscopy for biological application

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Abstract: Raman spectroscopy can provide information about chemical composition of the sample without destroying it. This advantage creates an opportunity to use Raman spectroscopy for label-free analysis of biological sample. In comparison to confocal scanning systems, which require long acquisition times, the Integral Field Spectroscopy (IFS) approach can create a snapshots of a large sample area and do imaging in real time. IFS is based on coding both spectral and spatial information in one exposure [1]. The acquired 2D image is then processed into a 3D hyperspectral cube. For sample excitation the lightsheet illumination was used [2]. This decreases scattering from out of focus regions and minimises exposure of the sample by excitation light, which is essential for biological samples investigation. In this work we discuss realisation, benefits and drawbacks of Integral Field Raman spectroscopy, methods for data processing, such as the conversion of 2D image to a 3D hyperspectral cube and a

mechanism for stitching several (50×50 pixel) hyperspectral images into a larger hyperspectral image. We did spectral analysis of the fingerprint area of various samples, which include plastic beads with different diameters and fixed cells.

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napari-live-recording: embedding camera acquisition in napari

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Abstract: Custom-built microscopes often require to control hardware objects directly. An high-complexity device group is cameras. Moreover, camera producers provide softwares which often lack the features needed to properly perform measurements as intended. The scientific community responded to this need by providing open-source solutions such as Micro-Manager[1], who pioneered the field, or python-microscope[2] to control cameras using a common interface. In this context, we present napari-live-recording, a plugin for napari[3]. The plugin aims to converge acquisition and analysis together, so to bring data analysis directly to the workbench for quick data evaluation. We aim to integrate a real-time image processing pipeline taking frames directly from cameras, showing results on image viewer or storing them using file formats such as TIFF and HDF5. Moreover, the plugin aims to converge existing camera control options into a single solution, by integrating the afore-mentioned Micro-Manager and python-microscope device layers within the plugin itself. The plugin architecture also allows scientists to integrate their own custom camera devices quickly and easily, minimizing the time to deploy new cameras or expanding the control over existing equipment. The plugin development is founded by the Chan-Zuckerberg Initiative through the "napari Plugin Foundation Grants (Cycle 2)" program.

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Adaptive optics for super-resolution microscopy in thick biological tissues

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Abstract: We are developing a fluorescence microscopy platform to observe interactions at the cellular and molecular level in thick biological tissues with a high isotropic spatial resolution. For that purpose, we use Image Scanning Microscopy (ISM) [1,2], a super-resolution technique improving the x-y resolution by imaging the PSF with a 2D array sensor. However, imaging deep into a biological sample induces severe aberrations deteriorating the PSF and the performances of ISM. To restore the PSF, we introduced an Adaptive Optics (AO) system consisting of a Deformable Mirror (DM) into the setup [3]. Outside of the microscope, our code successfully controlled the AO system through a feedback loop between the DM and a Shack-Hartmann WaveFront Sensor (WFS) to correct the wavefront. We implemented the DM alone into the commercial microscope, which led to a successful manual correction of fluorescent beads images, compensating aberrations intentionally introduced by a misadjusted correction collar of the objective. The data from ISM is expected to help us assess the spatially-dependent aberrations in the images to automate the correction process without using a WFS. 3D STED will be added to reduce the size of the PSF in the z direction and get closer to an isotropic resolution.

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Multidimensional data analysis of biological objects in nanoscale images

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Abstract: Image-based analysis of biological objects can be a powerful tool to compare, identify and find phenotypes in nanoscopy experiments [1,2]. However, batch effects resulting from experimental conditions can skew object representations [3]. Furthermore, they may hinder our reliance on expert features, which complicates the task of detecting subtler phenotypes [4,5]. In this project, we aim to develop an in-depth approach for the analysis of nanoscopy images, using the profiling of mitochondrial morphology in dopaminergic neurons derived from Parkinson's Disease (PD) patient stem cells as a case study. To do this, we exploit a robust quality control pipeline that can detect low-quality images and batch effects. Then, using a combination of single-object morphology feature

extraction, deep learning classification, and self-supervised learning paradigms, we show that the combined use of profile generation methods can identify features relevant for analysis at the nanoscale. Preliminary results show that batch effect correction methods are needed to obtain quantitatively comparable results, regardless of profiling type. When combined, these profiling methods reveal differences in mitochondrial morphology in PD. It suggests that integrating nanoscale information into biological object profiles can be a powerful tool to gain new insights about disease phenotypes.

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Unravelling the role of the vSNARE VAMP7 in membrane nanodomains

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Abstract: Neuronal cells display a high morphological complexity and morphogenesis during synaptic activity and learning. Tight regulation of intracellular tracking, to deliver cargos at various synaptic sites all over the dendritic tree, is an essential feature to ensure fast transmission. To successfully reach their destinations, vesicles need a precise and efficient trafficking. Among all the proteins that participate to intracellular tracking, SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are necessary for the very last step of a vesicle's journey: the membrane fusion. SNAREs are located both on vesicles (vesicular SNAREs, v-SNAREs) and target membranes (target SNAREs, t-SNAREs). Association of v-and t-SNARE in the so called SNARE complex, mediates membrane fusion by bringing both acceptor and donor membranes close enough. Moreover, previous studies show that VAMP7 play a role in the formation and stability of membrane nanodomains known to stabilize and facilitate the signalling of several proteins. These nanodomains are mainly composed of cholesterol and sphingolipids. Here, using epithelial cells and mature hippocampal neurons in culture and combining confocal and high resolution microscopy techniques such as STED and STORM, we are investigating the role of the vesicular SNARE VAMP7 in these nanodomains composition and what could be there role at synapses.

Quantum efficiency analysis of Raman signal enhancement by a cratered gold colloid

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Abstract: Investigation into the impact of gold nanoparticle morphology [1] on Raman signal enhancement was conducted through the development of a simulation using Ansys Lumerical Software. Specifically, a fixed spherical gold nanoparticle with an air cone crater was analyzed to induce a quadrupole mode within the nanoparticle.

The FDTD method was selected to discretize Maxwell's equations with minimal error, particularly when incorporating a PML. This method offers the convenience of selecting various wavelengths for the eigenvalues of frequencies.

The analysis of mode effective refractive index dispersion revealed that the propagation constant is minimal at a light wavelength of 785 nm [2] when the effective coefficient [3] approaches 0.1. Consequently, this resulted in the formation of a quadrupole mode, characterized by a radiation absorption-to-scattering ratio of approximately 0.9 by the nanoparticle. In the lower frequency range, a notable enhancement in the efficiency metric was observed [4].

The spatial distribution of fields in the proximity of a nanoparticle possessing a crater, under light propagation along the Z axis within the YZ plane, demonstrated field localization as a dipole [5], while a quadrupole mode manifested in the XY plane. Consequently, when estimating the electric and magnetic fields within a granule featuring a crater, consideration of quadrupole term is essential.

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Bioorthogonal labelling of surface receptors on living lymphocytes

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Abstract: There are several imaging methods for studying cell surface with molecular specificity, starting with conventional wide-field and confocal microscopies through super-resolution fluorescence microscopies to electron microscopies. Molecular specificity is achieved by labelling of target molecules. Antibodies (immunofluorescence) and fluorescence fusion proteins are commonly used for labelling of proteins. But these approaches have drawbacks for protein tracking in living cells due to their size/bivalency and poor fluorescence properties, respectively. Employing site-directed mutagenesis and optimised genetic code expansion [1], we have prepared mutants of CD4 protein suited for bioorthogonal labelling. Using Me-tet-Cy5 organic dye, we then compared this 'click' chemistry approach with antibodies and fusion to GFP for live-cell imaging and single molecule localisation microscopy (SMLM). Our results demonstrate that biorthogonal labelling outperforms immunofluorescence and fluorescence proteins in studies of nanoscopic receptor distribution [2]. Live-cell imaging of bioorthogonally labelled proteins provided results comparable to fusion with fluorescence proteins. However, our ultimate aim is to track CD2 and CD4 within the complex surface morphology of T cells. 'Click' chemistry enables the use of inorganic nanoparticles for a prolonged tracking of molecules. We are currently testing this approach.

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Advanced Imaging Projects at the Microverse Imaging Center

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Abstract: The Microverse Imaging Center is offering a full microscopy service for the scientists of Jena, in particular supporting the Excellence Cluster "Balance of the Microverse". The typical biological questions and microscopic probes of our users are very diverse: ranging from different microorganisms like bacteria, fungus, algae, to biological models like organs-on-chips and GUVs, we can also perform intravital imaging. The most widely used image modalities in our facility are ranging from confocal microscopy with or without super-resolution (AiryScan), Stochastic Optical Reconstruction Microscopy (STORM) and structured Illumination Microscopy (SIM). In this presentation, we will showcase several projects with an accent on high and super resolution.

Adaptation of microscopy for studying cell polarization

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Abstract: Cell polarization, i.e. a spatial difference in the structural organization such as of membrane molecules over the cell, plays an important role in cellular functions. An example is the polarization in epithelial cells, whose disruption by Ras gene mutations leads to cancerous developments. Still, the cell depolarization mechanism remains unknown. Here, we investigate polarized cells by means of advanced fluorescence microscopy and spectroscopy approaches, such as super-resolution STED microscopy, fluorescence correlation spectroscopy, and spectral imaging, to explore the differences between apical and basal membranes regarding lipid organization and Ras-lipid interactions. Importantly, we overcome the difficulties to measure polarized membranes with light fluorescence microscopy due to the photoselection effect, i.e., the preferential excitation of membrane dyes depending on their orientation with respect to the excitation light polarization. Our results are important to the study of polarized cell membranes using advanced fluorescence microscopy, and for the understanding of the role of Ras and its interaction with lipids.

The organization of organic/biominerals in the nanostructured 3D photonic crystals in insects' scales

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Abstract: The colorful patterns displayed by longhorn beetles Doliops similis (Cerambycidae:Laminae) are formed by opalescent photonic structures confined within their cuticular scales [1]. By using focus ion beam scanning electron microscope (FIBSEM), confocal laser microscope, Zeiss Elyra7 super-resolution microscope, complemented with Fourier-transform infrared spectroscopy (FTIR), Time-of-Flight SIMS (TOF-SIMS), x-ray photoelectron spectroscopy (XPS), we demonstrate that these needle-like scales contain a core-shell structure with a thin cuticular skin and an inner core of agglutinated amorphous calcium phosphate nanospheres (≈200 nm). The spheres assemble into hexagonally close-packed layers along the surface normal of the scale, producing hence a uniform and intense green reflection colour. The morphogenesis of the mineraloids nanoparticles in each scale and their self-assembly into opal-structures are unknown. We successfully located the chitin fibers in the scale by

chitin-binding proteins [2]. One of the hypotheses [3] is the arrangement of chitin and (lipo-)protein at the interface of the cuticular shell and mineraloid core mediates the packing of the nanospheres. To address this hypothesis, we wish to employ super resolution microscopy of intact scales in order to monitoring the organic molecules distribution in the skin and in the photonic crystals and investigate whether the organic molecular conformations and orientations are associated with the mineraloid crystallization.

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Unravelling SHP2-PD1 Interaction Dynamics using FRAP

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Abstract: Tumors can evolve to avoid recognition by the immune system. To this end, tumor cells often express the PD1 receptor ligand PD-L1 to suppress T cell activation and prevent tumor elimination [1,2]. In brief, upon PD1 engagement and phosphorylation, SHP2 phosphatase is recruited to PD1 [3]. This activates SHP2, reducing T cell activation through dephosphorylation of various signaling molecules [4]. Remarkably, the molecular and spatio-temporal mechanisms regulating SHP2 activity downstream of PD1 are poorly understood. We hypothesize that the cellular activity of SHP2 is regulated by phosphorylation-induced conformational changes and alterations in PD1 binding kinetics. We found five novel SHP2 phosphorylation sites and tested their effect on SHP2 dynamics and activity using TIRF and FRAP microscopy, as well as biochemical and functional assays. Mutating all known SHP2 phosphorylation sites reduced total enzymatic activity *in vitro* and inhibition of T cell activity. Furthermore, exchange rate and mobile fraction of SHP2 in interaction with PD1 was increased *in situ*. Currently, we are analyzing additional mutations and spatio-temporal changes to pinpoint the exact molecular mechanism. Our goal is to uncover novel T cell and PD1-specifc mechanisms controlling cellular activity of SHP2 that can be used as potential drug targets for future immunotherapies.

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Synaptoid or synaptic proteins in Tanycytes

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Abstract: Tanycytes are radial glia-like cells in the mediobasal hypothalamus. They form the blood-brain barrier in the median eminence and are involved in neuroendocrine axes and in central regulation of metabolism. Electron microscopy has shown that neurons contact tanycytes in synaptoid structures. The distribution and composition of synaptoids are unknown. In this study, we tried to characterize synaptoids using 3D-STED technology. In brains of wild-type mice or mice with genetically labeled tanycytes, we performed co-immunostaining of synaptic markers as a proxy to study synaptoids. The image acquisition was performed on a custom-built STED microscope (Abberior GmbH) using 3D-STED technology. First, we found that the presynaptic proteins bassoon and synaptophysin were closely apposed to the postsynaptic proteins shank2 and gephyrin in tanycyte. Second, synaptoids in tanycytes of median eminence had a diameter in range of 170 – 200 nm. And finally, we also compared these synaptoid puncta in median eminence and found that they were similar in. In summary, we found that both presynaptic and postsynaptic proteins of synaptoids were found in or in close vicinity of tanycytes.

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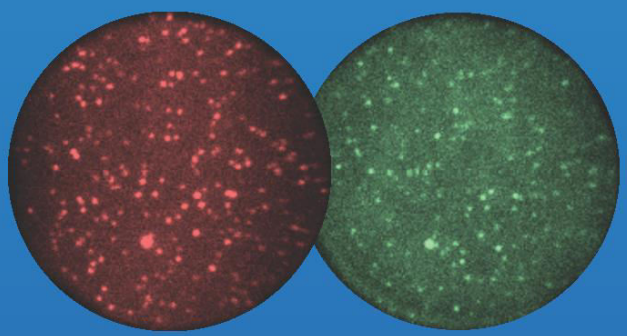

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