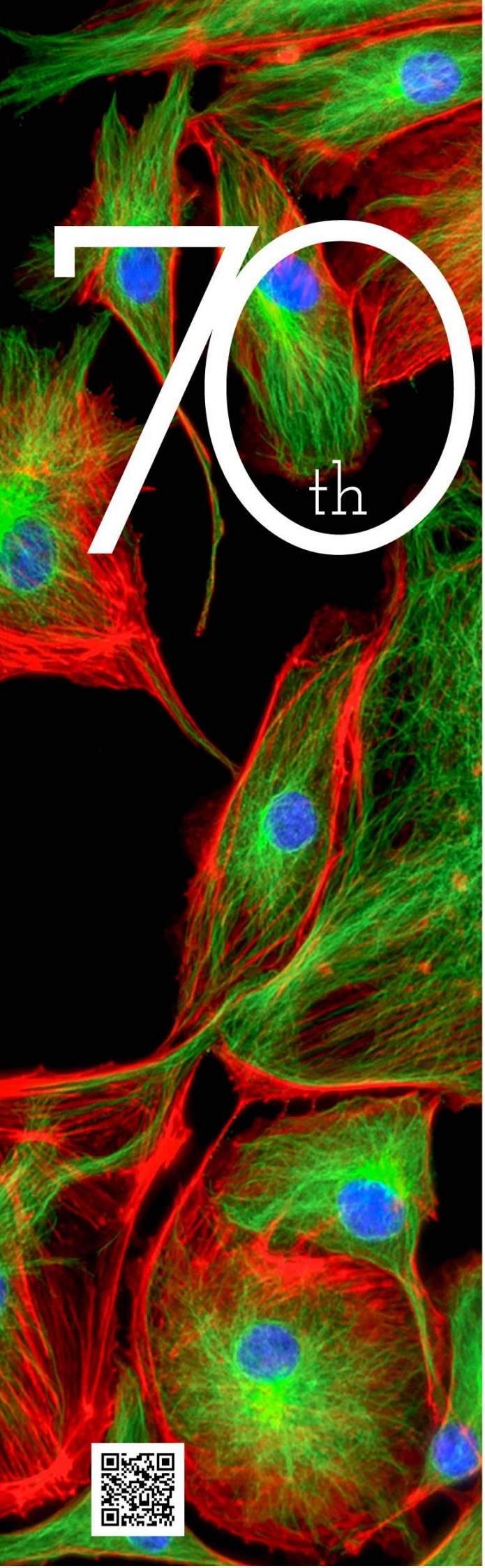

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Mosbacher Kolloquium

High-Resolution Imaging of Cells and Molecules

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ABSTRACTS

The structure of the nuclear pore complex and its degradation in the cellular context

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The nuclear pore complex (NPC) is a gigantic membrane protein forming a hole in the nuclear envelope. Due to its dimensions (~100 MDa in human) the elected method to determine the structure of the intact complex scaffold has been cryo electron tomography (cryo-ET). In addition, efforts to investigate the full NPC structure extracting it from its double membrane context have produced two structures in *S. cerevisiae*.

In our study we perform cryo-FIB-milling coupled to cryo-ET and subtomogram averaging to get the structure of the entire NPC scaffold in exponentially-growing cells.

Our structure shows significant differences in sub-complexes architecture to previous work emphasizing the importance of determining structures of membrane proteins in their native context.

In addition by means of correlative light and electron microscopy we shed the light on a quality control mechanism to degrade NPCs.

Online workflow platform for advanced light microscopy

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To understand the adaptive nature of organotypic heart cells in health and disease, subcellular membrane and protein structures are investigated. While **A**dvanced fluorescence **L**ight **M**icroscopy and superresolution **N**anoscopy (ALMN) provide more detailed visualization in cardiomyocytes, state-of-the-art imaging workflows and user needs are complex. Additionally, to optimize and reproduce imaging workflows for collaborative applications, modular workflows and image data registration are highly desirable. We have developed a flexible data-driven online platform to facilitate planning and management of ALMN projects for user groups.

Implemented as user operating models via the ALMN service project, online imaging requests were tailored to research questions through custom workflows. ALMN experts review each protocol and application including sufficient user training. For isolated cardiomyocytes and heart tissue, a variety of multi-color fixed and live cell ALMN workflows were successfully generated. Quantitative image

analysis tools and protocols were incorporated. Imaging metadata, data management and storage were unified as an online data repository.

In summary, we have developed an online ALMN platform for standardized imaging workflows including data storage and analysis. At the time of abstract submission, a total of 57 ALMN requests resulted in active workflows for 44 users from 13 research groups. Hence, the ALMN platform directly facilitates and integrates experimental approaches, user choices, instrument use, and training towards image analysis, publication, and data sharing.

A synthetic out-of-equilibrium morphogenic system

Presenting author: **Philippe I. H. Bastiaens**

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We reconstituted a Synthetic Morphogenic System (SynMS) by encapsulating a dynamic cytoskeletal microtubule (MT)-aster together with a light-actuated signaling system in a liposome with a deformable membrane. SynMS responds to light with self-organized morphological state transitions that manifest as self-amplifying membrane deformations generated by the MT-aster recursively interacting with the signaling system. We demonstrate that the perception and response to external light pattern stimuli are shaped by prior exposures and the ensuing morphological states. The interdependence between cytoskeletal dynamics, membrane shape, and signaling thus generated a minimal out-of-equilibrium 'life-like' system that mimics context dependent morphological responses of cells to external cues.

In Situ Architecture and Cellular Interactions of PolyQ Inclusions

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Expression of many disease-related aggregation-prone proteins results in cytotoxicity and the formation of large intracellular inclusion bodies. To gain insight into the role of inclusions in pathology and the in situ structure of protein aggregates inside cells, we employ advanced cryoelectron tomography methods to analyze the structure of inclusions formed by polyglutamine (polyQ)-expanded huntingtin exon 1 within their intact cellular context. In primary mouse neurons and immortalized human cells, polyQ inclusions consist of amyloid-like fibrils that interact with cellular endomembranes, particularly of the endoplasmic reticulum (ER).

Interactions with these fibrils lead to membrane deformation, the local impairment of ER organization, and profound alterations in ER membrane dynamics at the inclusion periphery. These results suggest

that aberrant interactions between fibrils and endomembranes contribute to the deleterious cellular effects of protein aggregation.

Structural flexibility of Synaptotagmin-1 probed by mass spectrometry and molecular dynamics

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A key step in neurotransmission is the fusion of synaptic vesicles with the presynaptic membrane. It depends on the intracellular level of Ca^{2+} and is tightly regulated by the calcium-sensor Synaptotagmin-1 (Syt-1). Syt-1 consists of a short transmembrane domain anchoring the protein in the vesicle membrane as well as two calcium-binding domains connected through an intrinsically disordered linker region. It has been described to interact with lipids on the presynaptic membrane, however, the functional role of this interaction is still debated.

We combine biochemical techniques, chemical cross-linking, ion-mobility and native mass spectrometry with computational analysis to characterise lipid interactions of Syt-1. First, oligomerisation of the soluble domains was tested by native MS revealing mostly a monomeric state. Subsequently, binding of Syt-1 to liposomes of defined lipid composition was assessed by flotation assays. Protein conformations of the liposome-bound population were then probed by XL-MS. To obtain direct information on ligand-induced conformational changes, we used IM-MS to investigate binding of specific lipid species. Furthermore, full-length Syt-1 including the transmembrane domain was incorporated into liposomes of different composition and analysed by XL-MS.

The distance restraints obtained from XL-MS were compared with molecular dynamics simulations of Syt-1 based on two high-resolution structures of an open and a closed conformation of the protein. Together with the IM-MS analysis, our data indicate that Syt-1 features a high flexibility that is modified by its specific interaction partners.

3D and Multicolor Live-cell Super-resolution Microscopy for Cell Biological Research

Presenting author: **Joerg Bewersdorf**

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Cell biological research relies heavily on the capabilities of light microscopes to resolve structures or processes of interest at the nanoscale [1]. My research group is developing super-resolution technology which achieve down to about 20 nm resolution in 3D and applies them to cell biological questions. We are, in particular, focused on improving the 3D resolution, speed, live-cell compatibility, throughput and depth penetration of STED and PALM/STORM microscopes [2, 3].

In my presentation, I will provide an overview of recent developments in my lab relevant for cell biological research. I will highlight our unpublished development of multicolor 4Pi-SMS imaging at 20

nm 3D resolution and live-cell applications of STED microscopy to investigate nanoscale dynamics of the endoplasmic reticulum [4].

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Analyzing the Effects of CK2 Inhibitors with an Indeno[1,2-b]indole Scaffold on Different Tumor Cell Lines by Live Cell Imaging and HPLC-MS/MS

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Human protein kinase CK2 is a ubiquitous and highly pleiotropic enzyme. Thus, CK2 is involved in a large number of cellular processes. However, its exact function is still not fully understood. Studies have shown that tumor cells contain an elevated amount of CK2 compared to normal cells. Furthermore, the protein kinase causes anti-apoptotic and proliferation enhancing effects in neoplastic tissues. Therefore, CK2 appears an interesting target for tumor therapy today[1,2].

Indeno[1,2-*b*]indoles represent one class of potent CK2 inhibitors, which exhibited promising results in *in vitro* experiments. Some of these small molecules are inhibiting the protein kinase with IC_{50} values in the nanomolar range [3,4]. To further consider the suitability as anti-cancer drugs, indeno[1,2-*b*]indoles were examined in cell culture experiments. This study focuses on the effects of indeno[1,2-*b*]indoles on different tumor cell lines. Live cell imaging was used to determine the influence of the CK2 inhibitors on the growth of A431, A549 and LNCAP cells. Furthermore, the induction of apoptosis in cancer cells after treatment with indeno[1,2-*b*]indoles was analyzed by western blot. For this purpose, the intracellular amounts of inactive procaspase-3 and active caspase-3 were determined. The caspase-3 activity was additionally analyzed *via* live cell imaging with the use of a fluorescent dye, which is only detectable when caspase-3 is active. Therefore, a higher number of apoptotic cells is indicated by an increase of fluorescence. Moreover, the uptake of indeno[1,2-*b*]indoles into tumor cells was determined. For this reason, the intracellular concentrations of these CK2 inhibitors were quantified via HPLC-MS/MS.

Bayer Pharmaceuticals PhD Prize:
Structural basis for coupling protein transport and N-glycosylation at the mammalian endoplasmic reticulum

Presenting author: **Katharina Braunger**

Asparagine-linked protein glycosylation is the most abundant post-translational modification of eukaryotic secretory proteins with crucial implications for protein folding, trafficking and functionality. Glycan transfer is catalyzed by the oligosaccharyltransferase (OST) - a complex of at least eight protein subunits which exists in two different isoforms (STT3A- and STT3B-OST) in higher eukaryotes. The process is tightly coupled to co-translational protein transport across or insertion into the endoplasmic reticulum membrane by the Sec61 protein-conducting channel. Formation of ribosome-translocon complexes (RTCs), including the OST has been described but the mode of OST-integration as well as the arrangement of OST subunits have remained elusive.

We used cryo-electron tomography to obtain structural evidence for exclusive incorporation of STT3A-OST in mammalian translocon-associated OST complexes. Additionally, we successfully determined the first high-resolution structure of an OST-containing RTC using single-particle cryo-EM. The structure has elucidated the subunit arrangement of mammalian STT3A-OST and includes molecular models for two core complex components. Furthermore, dissecting the interfaces between STT3A-OST and Sec61 or the ribosome, respectively, has enabled us to propose a model for isoform specificity of mammalian OST.

Our work substantially improves the understanding of mammalian N-glycosylation and the complex interplay of OST with the translation and translocation machineries.

Connecting live-cell imaging, cryofluorescence, and electron microscopy through microtechnology

Presenting author: **John A. G. Briggs**

MRC Laboratory of Molecular Biology, Cambridge, UK

We are interested in the structure and the assembly mechanisms of enveloped viruses such as HIV and influenza virus, and of cellular trafficking vesicles including clathrin and COPI coated vesicles. To study these systems we are combining cryo-electron tomography with computational image processing and other complementary techniques. In combination, these methods can reveal the structures of assembled protein complexes “in situ” - directly within their functional, native environments.

Elemental and Molecular Microscopy - Mass Spectrometric Imaging in the Life Sciences

Presenting author: **Rebecca Buchholz**

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Mass spectrometric bioimaging is a versatile tool to analyze endogenous and exogenous elements or molecules in different tissues. These techniques can complement classical histology or address targeted medical questions. In this study, the distribution of endogenous molecules like phospholipids, used to identify affected tissue after myocardial infarction, were analyzed. Additionally the target specific contrast agent (CA) Gadofluorine P was verified and quantified.

Coronary heart diseases like myocardial infarction (MI) are the most prevalent cause of death in the world. Magnetic resonance imaging (MRI) in combination with CAs could be a powerful approach to detect early clinical changes. Since most CAs are distributed systemically, it is of great importance to develop target-specific contrast agents.

Laser ablation coupled to inductively coupled plasma – mass spectrometry (LA-ICP-MS) was used to quantify Gd in murine heart tissue using external calibration with matrix-matched standards. Matrix-assisted laser desorption/ionization – mass spectrometry (MALDI-MS) was used to confirm the distribution of Gadofluorine P on a molecular level and to visualize tissue characteristics by endogenous molecules like phosphatidylcholine.

The combination of LA-ICP-MS and MALDI-MS as complementary techniques is a versatile approach to localize and quantify the Gd concentration originating from the administration of Gd-based CAs in infarctional heart tissue and healthy myocardium. A significant enrichment of Gd in infarctional tissue compared to healthy myocardium was found as well as higher accumulation in more advanced stages of MI. The distribution of Gd detected with LA-ICP-MS matches with the distribution of the Gadofluorine P ligand detected by means of MALDI-MS.

Connecting live-cell imaging, cryofluorescence, and electron microscopy through microtechnology

Presenting author: **Thomas P. Burg**

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In this talk, I will present recent advances of our group towards connecting live-cell imaging and electron microscopy (EM) through microtechnology-based methods for cryofixation and cryofluorescence imaging. Before entering the high vacuum of an electron microscope, biological samples generally need to be fixed chemically or frozen. Due to the time required for this preparation, it is difficult to correlate high-resolution EM images of cell structure with millisecond dynamics previously observed in the light microscope. We have been able to overcome this limitation by cryofixing cells and small model organisms directly in the light microscope through ultra-rapid in situ cooling. Ice crystallization is strongly suppressed by freezing at more than ~10^4 °C/s. Next, in order to image rapidly frozen samples by high numerical aperture cryofluorescence microscopy, we designed a new type of light microscope that allows immersion imaging below the glass transition of water (-135 °C). These technologies are compatible with conventional correlative light and electron microscopy workflows. We therefore expect that our platform will be of interest for studying a wide range of questions involving temporal relationships between cell stimulation, dynamic cell function, and structural alterations at the nanometer scale.

Automated Tools For Electron Microscopy

Presenting author: **Bridget Carragher**

The dramatic improvements in the progress of cryo electron microscopy (cryoEM) over the last five years has been accompanied by the adoption of a high level of automation. However, there are several automation challenges that remain to be addressed in the areas of specimen preparation, image acquisition, and analysis. In specimen preparation we need to address methods to ensure routine and robust preparation of particles embedded in a thin layer of vitreous ice. Our own group has developed a system that uses nanoliter droplets dispensed onto a self wicking grid that provides for a uniform and
thin layer of vitreous ice and improves the efficiency of data collection. More importantly it also appears to ameliorate adverse effects caused by proteins interacting at the airwater interface. In the area of image acquisition we anticipate a 10 fold increase in throughput assisted by a new generation of direct detectors. Additional instrument advances that would improve data throughput include developing faster and more stable cryo stages and further automating some of the steps required for high resolution imaging. Finally in the area of image analysis, the software continues to rapidly improve both in terms of speed and in being able to sort out highly heterogeneous populations of molecular complexes. All of these advances are being driven by the urgent needs of structural biologists and increasingly by the needs of pharmaceutical and biotechnology companies. The latter group in particular is driving the need for much higher levels of automation and much higher throughput.

Insight in the assembly mechanisms of Bax and Bak during apoptosis at the single molecule level

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The Bcl-2 family members Bax and Bak drive apoptotic cell death by assembling into oligomers that permeabilize the mitochondrial outer membrane (MOM).

Despite Bax and Bak have largely overlapping functions, whether they follow similar or different mechanisms of action during apoptosis is yet not known.
Here we use single particle imaging to quantify the real time dynamics of assembly of Bax and Bak in the mitochondria of apoptotic cells. Our data reveal divergent kinetics of oligomerization for these two proteins: while Bak oligomers assemble faster than Bax and reach equilibrium, Bax oligomers continuously grow overtime.
By complementary stoichiometry analysis in model membranes we have dissected the mechanism of assembly of Bak oligomers and revealed a concentration-dependent behavior for Bax and Bak oligomer formation.
These findings shed new light on the molecular mechanisms by which Bax and Bak proteins mediate MOM permeabilization during apoptosis and question the apparent redundancy of function between these key apoptotic proteins.

3D superresolution by supercritical-angle localization microscopy(SALM)

Presenting author: **Anindita Dasgupta**

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Anindita Dasgupta, Joran Deschamps, Jonas Ries

Single molecule localization microscopy (SMLM) has emerged as a powerful tool for imaging protein complexes at a resolution of tens of nanometers in both 2D and 3D. Popular approaches to 3D imaging engineer the microscope PSF to encode the position of single-emitters in its shape. However, such methods lead to a two times worse localization precision axially than laterally. Complex localization microscopy techniques, such as iPALM, can provide isotropic resolution at the cost of a complex instrument. In this work, we exploit supercritical-angle localization microscopy (SALM) to obtain 3D information with the prospect of an isotropic resolution and simple optics [1-3]. The method relies on measuring the supercritical angle fluorescence (SAF) generated by single molecule emitters close to the water-glass interface. Because the amount of SAF decreases exponentially with the distance of the emitter to the interface, its magnitude contains axial information. Since, SAF and undercritical angle fluorescence (UAF) propagate at different angles, they can be measured independently. Previous work showed the possibility to extract z-positions from their ratio. Here, we highlight the pitfall of current approaches and explore approaches to improve their resolution.

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Membranes as targets of Alzheimer's disease triggering amyloid beta peptides

Presenting author: **Norbert A. Dencher**

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Currently, Alzheimer's disease and related dementia affect about 47 million people worldwide. The highest risk factor for AD is age. Aβ peptides have been implicated in the late-onset of AD pathogenesis. In order to study cellular and organelle trafficking of Aß peptide, to identify its target(s), in our studies $Aß_{42}$ peptide monomers/small oligomers were externally applied to mammalian cells (human neuroblastoma cell line and rat oligodendroglia cell line). Monomeric/oligomeric peptides entered cells, as proven in our investigation by confocal fluorescence microscopy by employing fluorescently labeled $Aβ_{42}$ peptides. We were able to track in time and space the pathway of the Aß peptides from the outside of the cell across the plasma membrane to internal target membranes of specific organelles. In this way, for both cell lines, we did prove thatfluorescently labeled Aβ peptides initially co-localized with the plasma membrane and thereafter entered the cells and trafficked to

organelles, e.g., predominantly to lysosomes. The deep insertion of Aß peptides in lipid bilayers that subsequently induced membrane perturbations was verified by neutron scattering and fluorescence polarization. Aß affected numerous physiological cell parameters, such as ROS and ATP concentration, viability (necrosis/apoptosis), mitochondrial membrane potential and viscosity. Aß-induced alterations might cause neuronal cell death and AD pathology. The gained knowledge of Aß peptide–induced changes in the biochemical and biophysical properties of membranes contribute to our understanding of the pathology of Alzheimer's disease as well as being a clue for early diagnosis and efficient therapy.

Photon-free characterization of CMOS cameras removes bias from SMLM measurements

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We propose a photon-free characterization of CMOS cameras for gain, offset, noise and dark current in each pixel. We show that this approach can be effectively applied to experimental single-molecule localization microscopy (SMLM) data and specifically DNA-PAINT data where it removes localization bias.

CMOS cameras have evolved into a preferred camera architecture. Such detectors typically feature pixel-to-pixel variations in terms of noise, offset and gain. When being applied to SMLM, this effect can bias the localizations, but an appropriate characterization of the camera can restore the theoretically achievable uncertainties. So far, pixel-dependent dark current is often neglected. However, the localizations can be considerably biased in the vicinity of pixels featuring relatively high dark current. For measurements at long exposure times such as DNA-PAINT, this bias can well exceed the localization precision.

We demonstrate a photon-free approach that uses thermally generated electrons to characterize each camera pixel for gain, offset, noise and dark current. The thermally generated signal particularly plays a role for uncooled industry-grade CMOS cameras that are recently gaining popularity in the SMLM community.

We discuss the entire pipeline of an industry-grade CMOS camera characterization and how it removes the localization bias in experimental DNA-PAINT data. We furthermore demonstrate that our approach can be similarly applied dSTORM and PALM.

Structure and gating of Ryanodine receptor in lipid environment

Presenting author: **Rouslan G. Efremov**

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Concentration of calcium ions in cytoplasm of the cells regulates many physiological processes including transcription, cell cycle, apoptosis and muscular contraction. Calcium signalling in muscle contraction is mediated by ryanodine receptors, RyR, large 2.2 MDa homotetrameric ion channels that are primarily gated by changes in concentration of calcium ions in the cytoplasm and are regulated by multiple factors including ions, small organic molecules as well as via interactions with partner proteins. Here we used single particle cryo-EM to investigate the conformational transition in RyR1 from closed to open state for the protein reconstituted into lipid nanodiscs. We show that RyR1 reconstituted into lipid nanodiscs is stabilized in the open conformation when bound to the plant toxin ryanodine, but not in the presence of its physiological activators, calcium and ATP. Further, using ryanodine binding assays we show that membrane mimetics influence RyR1 transition between closed and open channel conformations. We observe that all detergents, including fluorinated detergent added to nanodiscs-reconstituted RyR1, stabilize closed state of RyR1. Further biochemical experiments support structural data and suggest optimal conditions for structural studies of RyR1 gating.

PARP1 regulates nucleolar-nucleoplasmic shuttling of WRN and XRCC1 upon genotoxic stress in a toxicant- and protein-specific manner

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The prime function of nucleoli is ribogenesis, however, recently several other, non-canonical functions have been identified, including a role in genotoxic stress response. Upon DNA damage, numerous proteins shuttle dynamically between the nucleolus and the nucleoplasm, yet the underlying molecular mechanisms are understood incompletely. Here, we investigated how PARP1 and PARylation contribute to genotoxic-stress-induced nucleolar-nucleoplasmic shuttling of two key genome maintenance factors, i.e. the RECQ helicase, WRN, and the base excision repair protein, XRCC1. Our work revealed that WRN translocates from nucleoli to the nucleoplasm upon treatment with the oxidizing agent H_2O_2, the alkylating agent 2-chloroethyl ethyl sulfide (CEES), and the topoisomerase inhibitor camptothecin (CPT). We demonstrate that after treatment with H_2O_2 and CEES, but not CPT, WRN translocation was dependent on PARP1 protein, yet independent of its enzymatic activity. In contrast, nucleolar-nucleoplasmic translocation of XRCC1 was dependent on both PARP1 protein and its enzymatic activity. Furthermore, by using gossypol, which inhibits PARP1 activity by disruption of PARP1-protein interactions, nucleolar-nucleoplasmic shuttling of WRN, XRCC1 and PARP1 was abolished, suggesting the involvement of further upstream factors. In conclusion, this study reveals a prominent role of PARP1 in the genotoxic stress-induced nucleolar-nucleoplasmic shuttling of genome maintenance factors in a toxicant- and protein-specific manner.

*Manuscript submitted for publication

3D-Imaging of Rabies Virus Infection of the Brain

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Rabies virus (RABV) is a highly neurotropic virus that spreads through the peripheral and central nervous system (CNS) via trans-synaptic spread by budding and entry at synaptic membranes. In the brain, infection of not synaptically connected cells is limited.

Restriction of RABV cell tropism to brain neurons may be determined at two levels: highly regulated release of RABV virions at synaptic membranes may limit infectious virus release to the extracellular space and strong innate immune responses by infected astrocytes may lead to abortive infection. Indeed, the latter has been demonstrated for lab adapted RABV strains. However, the potential of highly virulent field RABVs to infect non-neuronal cells *in vivo* and its contribution to virulence is unclear.

Here, using 3D immunofluorescence imaging of solvent-cleared brain tissue slices, we compared the cell tropism of attenuated vaccine virus SAD L16, lab-adapted challenge virus CVS-11 and two field RABV derived from Dog and Fox. Independent of the degree of attenuation or lab adaptation, all viruses mainly infected neurons. However, although much less frequent, field RABV infections also led to strong virus protein expression in non-neuronal, GFAP-positive astrocytes. In contrast, astrocyte infection was not detectable for lab-adapted RABVs. This, together with increased cytopathic appearance of lab strain-infected neurons, supports a model in which efficient replication of field RABV in astrocytes represents a backup immune evasion mechanism that may prevent local antiviral responses in a situation where virus release in not entirely restricted to synaptic membranes.

A proof-of-concept study of combined scanning ion conductance and stimulated emission depletion microscopy

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Understanding plasma-membrane related processes such as endo- or exocytosis or cell migration requires simultaneous observations of the plasma membrane dynamics and changes in the distribution of proteins of interest. Since the plasma membrane exhibits a three-dimensional ultrastructure with features with a size in the range of nanometers, a resolution beyond the diffraction limit of light is desirable.

To record physiological membrane dynamics reliably, the impact of the imaging tools on the sample have to be minimized. A bias-free method for measuring the membrane topography of living cells is scanning ion conductance microscopy (SICM). To sense non-conducting surfaces like the cell membrane, it utilizes the ion current through the opening of an electrolyte-filled glass capillary.

Because SICM can be operated under physiological conditions, it is suitable for long-term studies of membrane dynamics.

Here we present the first proof-of-principle recordings of correlated SICM and stimulated emission depletion (STED) microscopy. We recorded a STED image of actin and determined the nanostructure of the same, fixed HeLa cells with SICM. This is a first step towards the development of a combined STED-SICM instrument, which would provide the advantages of both techniques and allow investigating changes in topography and protein distribution in living cells with sub-diffraction resolution.

Multiscale Origami Structures as Interfaces for Cells (MOSAIC) – A versatile platform for the investigation of early cell signalling

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Surface-based bioanalytical methods are of utmost importance for fundamental research in molecular cell biology. We here describe a versatile method for the investigation of early cell signalling, Multiscale Origami Structures As Interface for Cells (MOSAIC)[1], which is based on the site-directed sorting of differently encoded, protein-decorated DNA nanostructures on DNA microarrays. Specifically, DNA origami nanostructures (DON) can be used as molecular pegboards for the precise arrangement of ligands for cell surface receptors with a lateral resolution of ~6 nanometers. DON constructs presenting the protein of interest can be employed to activate receptors in adhered cells. Their immobilization on glass surfaces is followed by adherence of cells and immunohistochemical analysis of transmembrane receptor activation.

With the help of the MOSAIC system we found that the nanoscale architecture of immobilized ligand-decorated DONs significantly affect receptor activation (e.g. EGF receptor) depending on both stoichiometry and the spatial arrangement of the ligands. Owing to its modularity, the MOSAIC technology can be used as tool for the investigation of various signalling pathways such as EGFR, integrin and ephrin.

The analysis of the MOSAIC system is performed by a variety of microscopy methods (fluorescence microscopy, TIRF, STORM, AFM). In combination with automated picture analysis, statistic evaluation and high throughput analysis can be achieved.

[1] Angelin, A. et al., 2015, *Angew Chem Int Ed* **54**, 15813

Mitoprotein stress in the cell: the HAP complex as an important regulator

Presenting author: **Carina Groh**

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Most of the 1000 proteins that are needed in mitochondria are encoded in the nucleus and synthesized on cytosolic ribosomes as a precursor form. To ensure the functioning of the organelle, these proteins need to be efficiently imported.

To study the cellular response to an overloaded mitochondrial import system in *Saccharomyces cerevisiae*, we use recombinant "clogger" proteins that reduce the number of available import sites. Our data show that cells downregulate the abundant enzymes of mitochondrial oxidative phosphorylation. The response is characterized by a reduced activity of a global transcriptional activator, the Hap2/3/4/5 complex, which controls the expression of many metabolic genes. In this way, cells adapt the synthesis of precursor proteins to the capacity of the translocation machinery to overcome the stress imposed by the accumulation of mitochondrial precursor proteins in the cytosol. Our data are providing fresh insight into a new way of cytosolic protection during proteotoxic stress.

Integrative imaging of membrane modulations in the course of virus-host interactions

Presenting author: **Kay Grünewald**

A mechanistic understanding of the complexity of structural cell biology in virus-host interactions requires a combination of tools and approaches. We apply electron cryo tomography (cryoET) in combination with complementary techniques to provide a comprehensive spatio-temporal picture of the functional interaction between viral protein complexes and cellular structures in the course of the infection. Understanding the entirety of a virus' 'life cycle' requires an understanding of its transient structures at the molecular level in their native cellular environment. Viruses serve moreover as dedicated tools to mine the molecular detail of cellular tomograms and to highlight uncharted mechanisms. Members from the herpesviruses, a family of enveloped large DNA viruses, constitute our main model systems. We are particularly interested on steps involving membrane remodelling and report here on the processes of herpesvirus entry (overcoming the plasma membrane) and herpesvirus nuclear egress (i.e overcoming the double membrane nuclear barrier). Along our biological questions, we constantly expand technologies and workflows and explore new combinations of approaches. The presentation will highlight some of these, including correlative microscopies, super-resolution fluorescence cryo microscopy, X-ray microscopy/tomography and proteomics.

Enlightening endocytic membrane dynamics

Presenting author: **Volker Haucke**[1,2,3]

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Biological membranes undergo constant remodeling by membrane fission and fusion to change their shape, to exchange material between subcellular compartments, or to spatiotemporally control cell signaling including neurotransmission at neuronal synapses. A prime example is the formation of endocytic vesicles by clathrin-mediated endocytosis CME) at the plasma membrane. CME, an essential

process for plasma membrane homeostasis and cell signaling, is characterized by a stunning heterogeneity in the size and lifetime of clathrin-coated endocytic pits (CCPs). If and how CCP growth and lifetime are coupled and how this relates to their physiological function is unknown. In my talk I will report on our most recent studies that combine automated tracking of CCP dynamics, electron microscopy, and functional rescue experiments to demonstrate that CCP growth and lifetime are closely correlated and mechanistically linked by the early-acting endocytic F-BAR protein FCHo2. FCHo2 assembles at the rim of CCPs to control CCP growth and lifetime by coupling the invagination of early endocytic intermediates to clathrin lattice assembly. Our data suggest a mechanism for the nanoscale control of CCP growth and stability that may similarly apply to other metastable structures in cells.

MINFLUX Nanoscopy: Superresolution post Nobel

Presenting author: **Stefan W. Hell**

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Max Planck Institute for Medical Research, Heidelberg, GER

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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[2] Göttfert, F., Pleiner, T., Heine, J., Westphal, V., Görlich, D., Sahl, S.J., Hell, S.W.Strong signal increase in STED fluorescence microscopy by imaging regions of subdiffraction extent. **PNAS** 114, 2125-2130 (2017).
[3] Balzarotti, F., Eilers, Y., Gwosch, K. C., Gynnå, A. H., Westphal, V., Stefani, F. D., Elf, J., Hell, S.W. Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes. **Science** 355, 606-612 (2017).
[4] Heine, J., Reuss, M., Harke, B., D'Este, E., Sahl, S.J., Hell, S.W. Adaptive-illumination STED nanoscopy. **PNAS** 114, 9797-9802 (2017).

A new approach to reconstruct dynamics from static super-resolution images to study clathrin-mediated endocytosis

Presenting author: **Philipp Hoess**

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As one of the super-resolution techniques, single-molecule localization microscopy (SMLM) has enabled new biological discoveries in the last decade. Highest spatial resolution in SMLM requires long acquisitions that typically last minutes to hours. Thus, it is required to chemically fix the sample so that the structure of interest is static and does not change during the acquisition.

However, many biological processes are highly dynamic and it is difficult to study them based on static super-resolution images. We aim to fill this gap by developing an approach to reconstruct dynamic information from individual snapshots. We acquire dual-color SMLM images and first align them in space and time using one color as reference structure. We then infer the structural distribution of a protein of interest from the second color channel. The approach includes high-throughput SMLM by automated microscopy and subsequent segmentation, alignment and averaging.

We apply this concept to study the spatial distribution of different proteins involved in clathrin-mediated endocytosis (CME) in budding yeast. This process is particularly well-suited to demonstrate our approach because it is well-known from conventional microscopy of live cells that the individual endocytic events progress with high spatial and temporal regularity. The dynamic reconstruction of the rearrangement of key players in this machinery will help us to understand their mechanistic function in CME.

Advanced Lattice Light-sheet Microscopy at a Bioimaging Core Facility

Presenting author: **Michael Holtmannspötter**

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Author(s):
Michael Holtmannspötter, Rainer Kurre, Jacob Piehler

Superresolution (SR) techniques amplified modern fluorescence microscopy by enhancing the achievable resolution beyond the diffraction limit. Multi-color Single Molecule Localization Microscopy (SMLM) in living cells combined with tailored spatiotemporal correlation analyses is a powerful tool to study the physiology and dynamics of cellular microcompartments. Structured Illumination Microscopy (SIM) is the method of choice for SR imaging at highest temporal resolution. All live-cell SR imaging projects need to optimize their imaging conditions to gain most information from the precious finite photon budget of each fluorophore. Applying these techniques to densely labeled three-dimensional dynamic samples requires widefield illumination techniques that allow for highest signal to noise levels with nearly no out-of-focus excitation. Lattice light-sheet microscopy (LLSM) developed by the group of Eric Betzig proved to allow for three-dimensional SR imaging techniques in thicker cells and even whole tissues [1].

As part of our core facility iBiOs, a clone of the Betzig LLSM was successfully set up in 2016. Since then, its superb features including high spatiotemporal resolution with low phototoxicity were applied to a variety of samples and organisms, ranging from single HeLa cells to whole drosophila embryos. We established structured illumination microscopy (SIM) as well as single molecule imaging using living HeLa cells. Currently, we are aiming to adapt SR techniques such as PALM, dSTORM and PAINT microscopy to our LLSM set-up to exploit the full potential of LLSM at a bioimaging facility.

1. Chen, B. C. *et al.* Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution. Science. **346**, 1257998 (2014).

Smart light sheet microscopy for you and me

Presenting author: **Jan Huisken**

Morgridge Institute for Research, Madison, WI

Discoveries in biology often depend on cutting edge technologies such as microscopy. Commercial microscopes are technically mature but often outdated and not well-tailored to the individual experiment's requirements. The best and latest microscopes are found in engineering and physics labs and the dissemination of the technology becomes a challenge. We have addressed this issue by developing the Flamingo, a modular, shareable light sheet microscope suited to a new model of scientific collaboration. Each microscope is customized for a given application, equipped to travel from lab to lab and providing widespread access to advanced microscopy. On the basis of scientific collaborations we provide these instruments so that the biologists can perform their experiments in their own lab. The Flamingo's modularity allows a variety of sample mounting techniques (tube, dish, gel, clearing, etc.). A horizontal arrangement offers single-sided illumination (L-SPIM, Huisken et al., Science), double-sided illumination (T-SPIM, Huisken [&] Stainier, Opt. Lett. 2007) and double-sided detection (X-SPIM, Schmid et al., Nat. Meth. 2013), all within the same framework. The hardware electronics are custom-built and offer remote control of the microscope. The system will evolve over time, driven by the community's feedback and input. We believe that the Flamingo is a powerful alternative to commercial solutions, open-source microscopes and conventional imaging facilities. More information can be found at www.involv3d.org/flamingo.

Bayer Pharmaceuticals PhD Prize:
Molecular characterization of public antibodies in human malaria

Presenting author: **Katharina Imkeller**

Katharina Imkeller[1,2], Hedda Wardemann[1]

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The humoral immune response plays a major role in acquired immunity towards Plasmodium falciparum (Pf), a causative agent of human malaria. Circumsporozoite protein (CSP) is the major surface protein of the pre-erythrocytic stage of Pf and some monoclonal antibodies directed against CSP have been shown to convey protection against infection. In this work we investigate the underlying molecular features of protective anti-CSP antibodies, which were previously not understood.

We characterize the human B cell response to the central repeat region of CSP by sequencing paired heavy and light immunoglobulin transcripts of single B cells from malaria exposed donors. Using a pairwise alignment tool that we developed to determine the similarity of antibody responses, we identified clusters of CSP specific antibodies with an amino acid sequence similarity of over 90% shared by different individuals. We recombinantly express these antibodies, characterize their binding behavior to CSP and test their Pf inhibition. Our data provide insights into the molecular mechanism

underlying the clonal selection and affinity maturation of human B cells expressing protective antibodies against CSP.

Interplay of Bax and Drp1 during apoptosis: A characterization by advanced microscopy

Presenting author: **Andreas Jenner**

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The permeabilization of the mitochondrial outer membrane (MOM) by the pro-apoptotic Bcl-2 family members Bax and Bak is considered a key step and a point of no return in the apoptotic pathway. This process is closely related to massive mitochondrial fragmentation by the mechanochemical GTPase Dynamin-related protein 1 (Drp1).

Previous results showed that, during apoptosis, Bax and Drp1 colocalize at discrete foci that likely correlate with ER/mitochondria contact sites supporting the assumption of a functional correlation between Bax and Drp1 during MOM permeabilization (MOMP).

However, the molecular mechanisms of their interaction and its relevance for efficient apoptosis signaling remain obscure.

Here we aim to elucidate such mechanisms by investigating i) how Bax and Drp1 interaction is affected by different mutant versions of Bax during MOMP; ii) the hierarchy of Bax and Drp1 recruitment to mitochondria under apoptotic conditions.

Furthermore, we use a single molecule approach to address the question whether Drp1 affects Bax oligomerization during MOMP. For this, we use stoichiometric analysis of Bax by advanced microscopy in membrane model systems and in cells to determine its oligomeric state in presence and absence of Drp1 during apoptosis.

These results will contribute to clarify the role of Drp1 in Bax-dependent MOM permeabilization and will provide new insights into the architecture of apoptotic foci at the MOM.

Dissecting structure, function and dynamics of molecular machines by single-molecule FRET microscopy: Translation initiation is regulated through modulation of the conformational dynamics of the DEAD-box protein eIF4A

Presenting author: **Dagmar Klostermeier**

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Translation initiation is a tightly regulated step in eukaryotic translation. Deregulation leads to altered gene expression, tumor formation and cancer. Initiation requires unwinding of secondary structures in the 5′-UTR of mRNAs by the DEAD-box helicase eIF4A, an RNA-dependent ATPase and ATP-dependent helicase. Both activities are stimulated by the translation factors eIF4B and eIF4G, and are linked to switching of eIF4A between open and closed conformations. Single-molecule FRET by confocal microscopy on eIF4A in solution shows that eIF4B, eIF4G and RNA modulate eIF4A activity by promoting formation of the closed state. Single-molecule experiments on surface-immobilized eIF4A by total internal reflection microscopy revealed that eIF4B and eIF4G jointly stimulate eIF4A activities by accelerating its conformational changes. The RNA substrate also influences eIF4A conformational dynamics: Short RNAs only partially stimulate the ATPase activity, and closing is rate-limiting for the conformational cycle. Longer RNAs maximally stimulate ATP hydrolysis and promote closing of eIF4A. However, the rate constants of unwinding do not correlate with the length of a single-stranded region preceding a duplex, but reach a maximum for RNA with a single-stranded region of only six nucleotides. We propose a kinetic model where eIF4B, eIF4G and the RNA substrate affect eIF4A activity and translation initiation by modulating eIF4A partitioning between futile, unproductive and productive cycles.

Expression of metabotropic glutamate receptors and cannabinoid receptors in hair cells of the cochlea

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In sensory tissues, such as the retina and the cochlea, highly specialized glutamatergic ribbon synapses are activated by light or sound. Their overexcitation causes an unphysiological high glutamate release, which is toxic for neurons and might induceapoptosis. While presynaptically localized metabotropic glutamate receptor (mGluR) types can directly inhibit glutamate release into the synaptic cleft, postsynaptic mGluRs inhibit glutamate release by themselves, or in concert with presynaptic cannabinoid receptors (CBRs). In the retina, the inhibitory function of mGluRs and CBRs is well known and associated with protective circuits, while in the cochlea similar mechanisms remain elusive. Here, we analyzed expression and localization of mGluRs and CBRs in the cochlea of adult mice and gerbils by PCR techniques and immunohistochemistry. For 3D Imaris reconstructions we used 2-Channel-STED-Microscopy and confocal microscopy. All known mGluR and CBR types were detected on the transcriptional level, except for the retina specific mGluR6. Antibodies recognizing a conserved epitope in mGluR2 and mGluR3, or immunesera directed against mGluR4, mGluR8 or CBR2 resulted in a punctate label in cochlear wholemounts, suggesting a synaptic localization. In addition, co-staining with a presynaptic marker (CtBP2) identified mGluR2/3 and CB2R at ribbon synapses of inner hair cells. Triple-staining of CtBP2 and mGluR2/3 with the postsynaptic marker PSD95 revealed postsynaptic localization of the receptors. In summary, we identified expression of several mGluR types and of CB2R at ribbon synapses of inner hair cells.

Vesicle-mediated nucleo-cytoplasmic transport of herpesvirus capsids

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Nucleo-cytoplasmic transport is supposed to occur exclusively through the nuclear pores. However, intra-nuclearly assembled 125nm herpesvirus capsids are too big to pass through to reach the cytosol for final virion formation. To overcome the nuclear envelope barrier herpesviruses use a vesicle-mediated pathway. Capsids bud at the inner nuclear membrane (INM) into the perinuclear space and acquire a primary virion envelope followed by fusion with the outer nuclear membrane.

Two viral proteins, designated as pUL34 and pUL31, form the nuclear egress complex (NEC) which mediates this budding process. pUL34 is a transmembrane protein which is targeted to the nuclear membranes and recruits the nucleoplasmic pUL31 to the INM forming the heterodimeric NEC. NEC oligomerization then drives membrane curvature and vesicle scission. Coexpression of both proteins in cells or model membranes results in vesicle formation indicating that no other viral or cellular protein is required.

Structures of the NECs from different herpesviruses as well as a multimodal imaging approach revealed how vesicles are assembled. However, how cargo (= capsid) is incorporated remained unclear. Structure-based mutagenesis identified a lysine (K242) at the membrane-distal NEC part as crucial. To test whether this is a direct interaction with the capsid, we replaced K242 by different other residues. Our data indicate that not charge but size and orientation of the side chain affects NEC-based vesicle formation.

Integrative analysis of the histone-modifying enzyme UTX in urothelial cancer combining fluorescence imaging spectroscopy with molecular biology

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The H3K27 specific demethylase UTX (KDM6A) is part of the COMPASS complex. The key components are the H3K4 mono-methyl transferases MLL2/MLL3, as well as the coactivators RbBP5, WDR5, ASH2L and DPY30, which are essential for the methyl transferase activity. UTX and MLL2/MLL3 proteins display a close functional cooperation since both H3K27 demethylation and H3K4 methylation are crucial for gene activation. Playing such an important role at genes and enhancers, MLL2, MLL3 and UTX are prone to a high mutation rate among other epigenetic factors, especially in the Kabuki Syndrome and in urothelial carcinoma (UC). Although the core components of the COMPASS complex are well known, information on COMPASS complex formation in UC, interaction sites between the components and the wider function of UTX dependent and independent of the COMPASS complex remain elusive. Therefore, we used an integrative approach combining molecular biology and

biophysical methods, such as Western Blot (WB), immunocytochemistry (ICC), immunoprecipitation (IP) and Multiparameter Fluorescence lifetime Imaging Spectroscopy (MFIS-FRET) to characterize the localization and interaction of UTX with the COMPASS core components in UC cell lines. Dependent on the mutation status of UTX, endogenous protein was detectable in the cytoplasm and/or in the nucleus of UC cells. Especially in stable transduced UTX cell lines, this protein predominantly localized to the nucleus in a special pattern. Co-IP experiments showed that RbBP5, a core component of the COMPASS complex, precipitates together with UTX. As somatic mutations in UTX and MLL genes often occur in UC, understanding the underlying biological and structural mechanisms could contribute to therapeutic approaches.

Quantitative Telomer Nanoscopy in Cardiomyocytes

Presenting author: **Tobias Kohl**

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Stabilization and protection of chromosome ends occurs through telomeric protein-DNA structures essential for genetic stability. In the heart with poor regenerative potential,telomere dysfunction activates the DNA damage response (DDR) promoting apoptosis, and cardiomyopathy. Telomer length and compaction are potential biomarkers of telomere function in heart disease, but difficult to analyze in mature cardiomyocytes, particularly in clinical biopsies.
We developed a telomere imaging approach for diagnostic application to clinical samples based on super-resolution microscopy of fluorescence in situ hybridization (FISH) of peptidenucleic acid (PNA) probes to telomeric DNA in cardiomyocytes (CMs). For imaging of thick and dense primary or stem cell derived CMs we used infrared STED nanoscopy and new FISH probes. STED imaging of intact mouse CM nuclei confirmed substantial variability among mammalian telomer sizes.Quantification of telomere sizes relies on photon-counting and bead calibration measurements, validated in HeLa cells for application to cardiac biopsies. In addition, Dynamic Intensity Minimum (DyMIN) STED measurements of sparse 3D telomeric FISH-signals are used for quantitative analysis.

A new reversibly switchable fluorescent protein for RESOLFT nanoscopy

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Reversible saturable optical linear fluorescence transition (RESOLFT) super resolution microscopy (nanoscopy) utilizes reversibly switchable fluorophores to overcome the diffraction barrier. Such fluorophores can be repeatedly toggled between a non-fluorescent off-state and a fluorescent on-state by irradiation with light. RESOLFT nanoscopy is particularly suited for recording living cells as this

approach requires low light doses for breaking the diffraction limit. Reversibly switchable fluorescent proteins (RSFPs) are well established fluorophores for RESOLFT live cell imaging. They operate in various modes: The so called negatively switching RSFPs represent the majority of RSFPs. Upon excitation these RSFPs either fluoresce or are transferred into the non-fluorescent off-state. From the off-state they can be switched back into the on-state by irradiation with light of a different wavelength. Because fluorescence excitation and switching off are competing processes the number of photons that are emitted from a single molecule in its on-state is limited, which can be a challenge in nanoscopy. Positively switching RSFPs circumvent this limitation and are, in theory, better suited for RESOLFT nanoscopy as the excitation wavelength also switches the protein from the off- to the on-state. Currently, only few applications for positively switching RSFPs have been reported. One reason for this is the poor performance of the available RSFPs. By combining mutagenesis and various screening approaches we have engineered a new RSFP based on the positively switching RSFP Padron [1] with properties specifically tailored to the requirements of RESOLFT nanoscopy.

[1] Andresen, M., et al. (2008). Nat Biotechnol **26**(9): 1035-1040.

Mitochondrial precursor proteins control the mitotic cell cycle

Presenting author: **Lena Krämer**

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Most mitochondrial proteins are synthesized in the cytosol and subsequently imported into mitochondria. A functional mitochondrial protein import is essential for cell survival. Whereas the processes by which mitochondrial precursor proteins are translocated into mitochondria have been examined in detail, much less is known about the consequences of import failure.

To study how cells deal with the accumulation of mitochondrial precursor proteins, we expressed mitochondrial "clogger" proteins in yeast. These recombinant proteins reduce the number of available mitochondrial import sites and overcharge the import system which inhibits cell growth. Our data show that the accumulation of mitochondrial precursor proteins in the cytosol leads to the induction of the transcription factor Rpn4 which upregulates the ubiquitin-proteasome-system. Surprisingly, deleting Rpn4 seems to restore precursor-induced growth defects. Rpn4 apparently triggers a specific arrest of the cell cycle and, hence, is part of a cellular control system that coordinates mitochondrial biogenesis with cell division.

The Molecular Mechanism of λN-dependent processive transcription antitermination

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Transcription by the bacterial RNA polymerase (RNAP) is a tightly regulated molecular event, comprising of three phases: initiation, elongation and termination. The λN factor from lambdoid phages can render the elongationg RNAP termination-resistant. This process has been termed λN-mediated processive antitermination. Here we present a 3.7 Å cryo-electron microscopic structure of a complete transcription antitermination complex consisting of RNAP, DNA, RNA, including the *nut* site, λN, NusA, NusB NusE and NusG. Due to intrinsic disorder, λN can act as a multiprotein/RNA interaction hub, which rearranges several elements of the complex. Based on the structure and on results from structure-guided mutagenesis, we hypothesize that λN mounts a multipronged strategy to reprogram the transcriptional machinery in several ways: (1) it repositions NusA and remodels the RNAP β flap tip, preventing accommodation and stabilization of regulatory RNA hairpins in the RNA exit tunnel, (2) it positions NusA and NusE to provide a surface for the NusG CTD, counteracting ρ-dependent termination, (3) it cooperates with NusG to stabilize the upstream DNA, preventing backtracking, and (4) it invades the catalytic cavity, stabilizing the DNA:RNA hybrid and the active state of RNAP. Our findings show how λN reprograms the transcriptional machinery via a combination of global and local remodeling events. Furthermore, the near-atomic resolution structure might in the future guide the design of peptides, that interfere with bacterial transcription.

Feodor Lynen Lecture:
Mechanistic insights from high-resolution cryoEM structures of ATP synthases

Presenting author: **Werner Kühlbrandt**

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With the ongoing resolution revolution in electron cryo-microscopy (cryoEM; Kühlbrandt, 2014), large and dynamic membrane protein complexes have become accessible to high-reso[-]lution structural studies. We have used single-particle cryoEM to determine the structure of the complete, monomeric ATP synthase (cF_1F_o) from spinach chloroplasts (Hahn et al, 2018), and of the dimeric mitochondrial F_1F_o ATP synthase (mtF_1F_o) from the green alga *Polytomella* (Allegretti et al, 2015; Klusch et al, 2017), both at around 3 Å resolution. Bound nucleotides with their coordinating Mg ions and water molecules are resolved in cF_1. The two-domain subunit *δ* of cF_1F_o (*OSCP* in mitochondria) joins the three *α*-subunits of the F_1 head to the peripheral stalk in three different ways. Three resolved rotary states of cF_1F_o indicate that the peripheral stalk flexes to store torsional energy, whereas subunit *γ* of the central stalk works as a non-flexible rigid body. In both mitochondria and chloroplasts, subunit *a* in the membrane-embedded F_o motor forms two aqueous channels to conduct protons to and from the protonation sites on the *c*-ring rotor that powers ATP generation. The channels and the polar and charged sidechains that define them in the hydrophobic membrane interior are conserved over an evolutionary distance of around 1.5 billion years (Kühlbrandt, 2019). The F_o motor assembly with its hairpin of long, membrane-embedded subunit *a* helices adapts equally well to the 10-subunit *c*-ring of mtF_1F_o and the 14-subunit *c*-ring of cF_1F_o. Electron cryo-tomography of chloroplast thylakoids indicated that cF_1F_o is always monomeric, whereas all mtF_1F_o dimers form rows that impose high local membrane curvature on the inner membrane (Davies et al, 2012; Mühleip et al, 2016; 2017). When reconstituted into proteoliposomes, ATP synthase dimers assemble into rows spon[-]taneously, inducing high local membrane curvature as in mitochondria (Blum et al, 2019; Kühlbrandt, 2019).

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Interference with actin-based bacterial dissemination by the human host defense protein hGBP1

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Several species of invasive bacteria including the human-adapted, enteric pathogen *Shigella* enter host cells via endocytosis or phagocytosis and subsequently escape from an intracellular vacuole into the host cell cytosol. Survival within the cytosolic milieu drove the evolution of specific niche adaptations. One adaptation commonly found amongst intracytosolic bacterial pathogens such as *Shigella* is the ability to hijack the host's actin polymerization machinery in order to generate force for intracytosolic movement and the invasion of neighboring host cells. This actin-based motility enables *Shigella* to spread within the colonic epithelium while simultaneously avoiding extracellular immune mechanisms such as antimicrobial peptides or neutrophils. Host defense against *Shigella* is therefore critically dependent on immune defense programs executed by the infected cell itself. One such cell-autonomous defense program directed at *Shigella* is executed by the dynamin-related GTPase human guanylate-binding protein 1 (hGBP1). Our lab and others reported that hGBP1 binds to *Shigella* and blocks bacteria from utilizing the host actin polymerization machinery for intracytosolic motility and cell-to-cell spread. The mechanisms underlying this potent hGBP1-mediated defense program is currently unknown and represents a critical gap in knowledge. We observed that hGBP1 blocks recruitment of host factors important for actin tail formation to the poles of *Shigella*. In ongoing biochemical and biophysical studies we are investigating how hGBP1 achieves to block recruitment of

the actin polymerization machinery to *Shigella*. Further, we are investigating if this potent hGBP1-mediated host defense is also directed at other microbial pathogens which use actin-based motility for dissemination.

NudC – a new player in WIPI-mediated autophagy

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Autophagy is a highly conserved lysosomal degradation pathway in eukaryotic cells that is upregulated in response to stress stimuli. A double membrane vesicle called the autophagosome mediates the degradation of cytoplasmic components. A crucial step of the membrane rearrangements leading to the formation of the autophagosome is the generation of PtdIns(3)P. Subsequently the human WIPI proteins are recruited to fulfill essential functions as PtdIns(3)P effector proteins. NudC, a conserved microtubuli-associated protein was recently identified in a proteome analysis of the human WIPI proteins *(Bakula et al., 2017)*. In this study it was confirmed that NudC interacts with WIPI1, WIPI2 and WIPI4 *(Bakula et al., 2017)*. Subsequent characterizations revealed that NudC is a new autophagy inhibitor. The inhibitory function of NudC is demonstrated by the results that a reduced NudC protein expression (1) increases the number of WIPI1-, WIPI2-, and LC3- positive autophagosomes, (2) reduces the levels of p62 in human tumor cells, (3) increases the rate of long-lived protein degradation and lastly (4) increases the number of autophagosomes observed in electron microscopy analysis. Likewise, overexpression of NudC reduces the number of autophagosomes. We suggest that NudC regulates WIPI-mediated autophagy, perhaps by preventing WIPI proteins from localizing to PtdIns(3)P-enriched membranes.

Molecular strategies for regulating microtubule dynamics

Presenting author: **Carolyn Moores**

Many aspects of eukaryotic cell function depend on the microtubule cytoskeleton. Microtubules are built from tubulin dimers and their intrinsic GTPase drives polymer dynamics. In vivo, an array of microtubule-associated proteins (MAPs) maintains tight control of microtubule stability and plasticity. However, the extent to which MAPs read and respond to the tubulin GTPase cycle and/or override it remains unclear. Our recent work has been aimed at elucidation of regulatory mechanisms of microtubule dynamics using cryo-electron microscopy and near-atomic resolution structure determination.

Chip-Based Sensing of the Release of Unprocessed Cell Surface Proteins in Response to Stress

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Motivation: To study the possibility that components of eukaryotic plasma membranes are released under certain (patho)physiological conditions, a chip-based sensor was developed for the detection of cell surface proteins, which are anchored at the outer leaflet of eukaryotic plasma membranes by a covalently attached glycolipid, exclusively, and might be prone to non-enzymic release.

Method: Those unprocessed glycosylphosphatidylinositol-anchored proteins together with associated phospholipids become specifically captured and detected by the sensor leading to changes in phase and amplitude of surface acoustic waves propagating over the chip surface.

Results: They were found to be released from rat adipocyte plasma membranes immobilized on the chip, dependent on the flow rate and composition of the buffer stream, and to be present in the incubation medium of adipocytes, in correlation to the cell size, and in rat serum, in correlation to genotype and body weight, as well as in human serum.

Conclusion: The chip-based sensing for unprocessed cell surface proteins reveals the inherent susceptibility of plasma membranes for (endogenous/exogenous) stress, which may be useful for monitoring of (metabolic) disease states.

Combining Convolutional Neural Networks and Fitting Algorithms for High Density Single Molecule Localisation Microscopy

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Key to super-resolution microscopy based on single molecule localisation is the acquisition of many thousand frames, each containing only a subset of the fluorophores. Reconstructing super-resolution images requires the fitting of a PSF model (e.g. a Gaussian or cubic spline model) to the experimentally observed data . While in the past comparably simple single emitter fitter operating on a defined subregion were present, the increasing need for faster imaging acquisition led to the advent of high-density fitters based on iterative single emitter or multi-emitter approaches. In recent days, many algorithms feature modern machine learning algorithms utilising (convolutional) neural networks which require prior training and can then output localisations non-iteratively (in a single forward pass).

When initialisation is bad or model selection is hard, classical fitting algorithm can get easily stuck in local minima and fail. We combine the best of both worlds, featuring a (C)NN as an initialisation and

model selector and feed this information in a medium density fitting algorithm which in principle can achieve optimal precision.

We investigate the accuracy improvement of the two-fold reconstruction as well as the reduction of training time for the (C)NN.

Rotary substates of ATP synthase by cryo-EM reveal the basis of flexible F_1-F_0 coupling

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Author(s):
Bonnie J. Murphy, Niklas Klusch, Özkan Yildiz, Werner Kühlbrandt

F_1F_o-ATP synthases play a central role in cellular metabolism, making the energy of the proton-motive force across a membrane available for a large number of energy-consuming processes. We determined the single-particle cryo-EM structure of active dimeric ATP synthase from mitochondria of *Polytomella sp*. at 2.7- 2.8 Å resolution. Separation of 13 well-defined rotary substates by 3D classification provides a detailed picture of the molecular motions that accompany *c*-ring rotation and result in ATP synthesis. Crucially, the F_1 head rotates along with the central stalk and *c*-ring rotor for the first ~30° of each 120° primary rotary step. The joint movement facilitates flexible coupling of the stoichiometrically mismatched F_1 and F_o subcomplexes. Flexibility is mediated primarily by the interdomain hinge of the conserved *OSCP* subunit, a well-established target of physiologically important inhibitors. Our maps provide atomic detail of the *c*-ring/*a*-subunit interface in the membrane, where protonation and deprotonation of *c*-ring *c*Glu111 drives rotary catalysis. An essential histidine residue in the lumenal proton access channel binds a strong non-peptide density assigned to a metal ion that may facilitate *c*-ring protonation, as its coordination geometry changes with *c*-ring rotation. We resolve ordered water molecules in the proton access and release channels and at the gating *a*Arg239 that is critical in all rotary ATPases. We identify the previously unknown *ASA10* subunit and present complete *de novo* atomic models of subunits *ASA1-10*, which make up the two interlinked peripheral stalks that stabilize the *Polytomella* ATP synthase dimer.

Dopaminergic Cells are Protected from Toxic Insults and Show Enhanced Tyrosine Hydroxylase Activity by Cellular Delivery of a Fusion Protein Composed of the Transcription Factor Nurr1

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Parkinson's disease is caused by the degeneration of dopaminergic neurons in the *substantia nigra*. The development and maintenance of dopaminergic cells depend on the orphan transcription factor nuclear receptor-related 1 protein (Nurr1). Cellular delivery of proteins can be achieved after fusion with the detoxified N-terminal domain of lethal factor (LFn) which is derived from *Bacillus anthracis*.

In this study, we generated a fusion protein named HS-LUNN1 consisting of LFn fused to wild type ubiquitin, a nuclear localization sequence along with human Nurr1. After bacterial expression, isolation, and purification of HS-LUNN1, its cellular uptake and biological activity were shown by a luciferase reporter assay utilizing the tyrosine hydroxylase promotor, which becomes activated by Nurr1 protein binding. In addition, the expression of tyrosine hydroxylase was increased by cellular delivery of HS-LUNN1 into SHSY-5Y cells. Furthermore, cellular application of HS-LUNN1 protected these cells from the toxic insult of 6-hydroxydopamine (6-OHDA). Finally, domain variations of HS-LUNN1 were analyzed regarding their efficiency in cellular uptake.

In summary, cellular delivery of HS-LUNN1 increased tyrosine hydroxylase activity and protects from cell death induced by 6-OHDA. These results may advance developments of a protein-based therapy treating Parkinson's disease.

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Paliga, D., Raudzus, F., Leppla, S.H., Heumann, R., Neumann, S.: Lethal Factor Domain-Mediated Delivery of Nurr1 Transcription Factor Enhances Tyrosine Hydroxylase Activity and Protects from Neurotoxin-Induced Degeneration of Dopaminergic Cells. Molecular Neurobiology, 2018, DOI: 10.1007/s12035-018-1311-6

Biological cells and tissues studied by holographic X-ray microscopy and tomography

Presenting author: **Jan-David Nicolas**

X-rays deeply penetrate matter and thus provide information about the functional (interior) architecture of complex samples, from biological tissues and cells to novel composite materials. However, this potential of hard x-rays in view of penetration power, high spatial resolution, quantitative contrast, and compatibility with environmental conditions has to date not been fully developed, mainly due to significant challenges in X-ray optics. With the advent of highly brilliant radiation, coherent focusing, and lensless diffractive imaging this situation has changed. We show how hard X-rays focused to nanometer spot sizes can be used for scanning as well as for full field holographic X-ray imaging of biological samples [1]. The central challenge of inverting the coherent diffraction pattern will be discussed for holographic techniques [2] and ptychography [3,4].

By scanning the sample through the focused X-ray beam and recording diffraction patterns in each scan point, structural parameters can be mapped throughout the cell or histological section [5], yielding a 'diffraction contrast' image that can show how nanometer-sized structures can vary within the tissue. As an example, we address the sarcomeric organization in heart muscle cells (cardiomyocytes) [6,7], and show how the sarcomere organization evolves and differs between different cell types and maturation states. As a multi-scale approach, we then discuss sarcomeric structure in heart tissue sections [8], and then finally present phase contrast tomography reconstructions of an entire mouse heart. A similar multi-scale approach is outlined for the case of neuronal tissue [9].

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Single molecule imaging reveals a role of a novel phosphorylation site of the stress granule organizer G3BP1 in liquid phase mobility

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Stress granule (SG) formation is driven by phase separation into liquid-like droplets, creating a highly dynamic microcompartment. Regulation of phase transition and the material state of SGs is thought to be modulated by multivalent RNA-binding proteins (RBPs), which can be posttranslationally modified, possibly leading to a change in composition and function of SGs.

To identify potential phosphorylation sites that affect the function of individual SG components, we performed a comparative phosphoproteomic analysis after experimentally induced stress in model neurons. We identified Ser253 as a novel phosphorylation site of the SG organizer G3BP1, which is exclusively phosphorylated after stress induction. To analyze the impact of phosphorylation at this site on diffusion and interaction within stress granules (SGs), we introduced a phosphoblocking mutation (S253A) in Halo-tagged G3BP1 and performed single molecule tracking experiments. We observed alternating binding in nanocores and anomalous diffusion in the liquid phase of SGs with similar characteristics for G3BP1 wildtype and the S253A mutant. Remarkably, we observed a large decrease of anomalous diffusion of the phosphoblocking mutant in the liquid phase indicative for a higher viscosity of liquid droplets. Our data provide direct evidence that phosphorylation of SG components modulates diffusion in the liquid phase, which may influence the material state of SGs.

Advances in optoacoustic imaging for biomedical applications

Presenting author: **Vasilis Ntziachristos**

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Optical imaging is unequivocally the most versatile and widely used visualization modality in the life sciences. Yet it has been significantly limited by photon scattering, which complicates the visualization of tissue beyond a few hundred microns. For the past few years, there has been an emergence of powerful new optical and optoacoustic imaging methods that offer high resolution imaging beyond the penetration limits of microscopic methods. The talk discusses progress in multi-spectral opto-acoustic tomography (MSOT) and mesoscopy (MSOM) that bring unprecedented optical imaging

performance in visualizing anatomical, physiological and molecular biomarkers. Advances in light technology, detection methods and algorithms allow for highly-performing visualization in biology and medicine through several millimetres to centimetres of tissue and real-time imaging. The talk demonstrates implementations in the time and frequency domain, showcase how it is possible to accurately solve fluence and spectral coloring issues for yielding quantitative measurements of tissue oxygenation and hypoxia and demonstrate quantitative *in-vivo* measurements of inflammation, metabolism, angiogenesis in label free mode.

Bio:
Vasilis Ntziachristos Ph.D. is Professor of Medicine, Professor of Electrical Engineering and Director of the Chair for Biological Imaging (CBI) at the Technical University of Munich, Director of the Institute for Biological and Medical Imaging (IBMI) at the Helmholtz Zentrum Munchen and Director of Bioengineering at the Helmholtz Pioneering Campus. He has received the Diploma in Electrical Engineering and Computer Science from the Aristotle University of Thessaloniki, Greece and the M.Sc and Ph.D. degrees in Bioengineering from the University of Pennsylvania in Philadelphia PA. Prior to his current appointment he was faculty at Harvard University and the Massachusetts General Hospital. Professor Ntziachristos is the Editor of the journal Photoacoustics, regularly Chairs in international meetings and councils and has received numerous awards and distinctions, including the Gold Medal from the Society for Molecular Imaging (2015), the Gottfried Leibnitz prize from the German Research Foundation (2013), the Erwin Schrödinger Award (2012) and was named one of the world's top innovators by the Massachusetts Institute of Technology (MIT) Technology Review in 2004.

Image-based 3D structural analysis of FtsZ networks in Physcomitrella patens and feature-based network classification via machine learning

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Many proteins, including proteins of cytoskeleton, function in the form of complex polymeric networks. 3D morphological and connectivity features of such networks are informative for the function of the respective proteins. In this project we have developed a computational pipeline for semi-automatic quantitative analysis and comparison of protein network morphologies based on 3D CLSM images of FtsZ networks in the chloroplasts of *P. patens*. The method is capable of detecting subtle differences in network morphology between two closely related isoforms of FtsZ family. Developing on this ground, we are currently building machine-learning-based classification algorithms which will be able to assign random images of FtsZ networks to the correct isoform class based on the specific 3D morphological features of the networks. Unbiased quantitative comparison/classification of protein networks can enable accurate analysis of influences of various experimental conditions (such as mutagenesis, drug treatment, or transgene expression) on protein network morphology.

A 192-Heme Electron Transfer Network in the Hydrazine Dehydrogenase Complex

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Anaerobic ammonium oxidation (anammox) is a major process in the biogeochemical nitrogen cycle in which nitrite and ammonium are condensed to dinitrogen gas and water, via the highly reactive intermediate hydrazine [1]. To date, it is still unknown how anammox organisms convert the toxic hydrazine into nitrogen and harvest the extremely low potential electrons (-750 mV) released in this process. Here, we report the crystal structure and cryo-EM structures of the responsible enzyme, hydrazine dehydrogenase (HDH) [2]. HDH is a 1.7 MDa multiprotein complex containing an extended electron transfer network of 192 heme groups spanning the entire complex. This unique molecular arrangement suggests a potential mechanism for the storage and release of the electrons obtained from hydrazine conversion, the final step in the ubiquitous important anammox process.

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Single-Vesicle Microscopy of Reconstituted Model Membranes

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Due to the amphipathic nature of membrane proteins, their reconstitution into model membranes is an essential approach for the investigation of individual features and activities of specific cell membrane components under both native like and chemically-defined conditions. Established model-membrane systems used in ensemble average measurements are limited by sample heterogeneity and insufficient knowledge of lipid and protein content, which prevents quantitative analysis of vesicle properties, substrate transport, and their correlation with protein activity. The use of microscopy based techniques reveals the connection between such properties on a single-vesicle level, thereby overcoming these drawbacks. Here we show that single vesicles containing fluorescent membrane proteins and lipid markers can be immobilized, imaged by confocal microscopy, and quantified by image analysis. Application of membrane-impermeant quenchers allows for the determination of vesicle lamellarity, protein orientation, and reconstitution efficiency in the immobilized proteoliposomes. We will extend this approach for identification of factors affecting protein orientation during reconstitution and improve the analysis of membrane-protein activity in a well-defined environment.

Resolving the nanopathology and rescue of Junctophilin-2 clustering in atrial cardiomyocytes

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Atrial dysfunction is highly prevalent and aggravates heart failure, yet the molecular and cellular mechanisms remain unclear. Junctophilin (JP2), a tail-anchored protein of the sarcoendoplasmic reticulum, which stabilizes membrane contact sites (MCSs), is significantly decreased in failing hearts. We investigated the role of JP2 in healthy and diseased atrial cardiomyocytes (AM) by superresolution STED microscopy including live-cell imaging. In AMs, dual-color STED-IF identified an atria-specific subcellular mechanism of differential JP2 expression in large clusters co-localized with ryanodine receptor (RyR2) Ca2+ release channels at MCSs with axial tubule membrane invaginations. In hypertrophied AMs, JP2 expression was decreased, RyR2-channel clustering disrupted and Ca2+ release disturbed. Selective shRNA-mediated JP2 knock-down confirmed this loss-of-function in vivo. In contrast, JP2 overexpressing mice showed preserved atrial function, augmented RyR2-channel clustering and strikingly, de novo biogenesis of a MCS supercomplex with functional Ca2+ release in living AMs. Together, our data identify a new model of subcellular JP2 clustering and atrial dysfunction, which is rescued by JP2 overexpression.

Understanding cilia assembly and transport with CLEM and cryo-EM

Presenting author: **Gaia Pigino**

Abstract not submitted

Unravelling Mechanisms of Glycolipid-dependent Endocytosis

Presenting author: **Robert Pilz**

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Sphingolipids (SLs) are bioactive membrane molecules, which are expressed in a cell-specific and cell differentiation-dependent manner. They fulfil diverse functions in the cell including signalling and cell fate decisions. Recent work of I. Morace et al.[1] revealed the involvement of complex glycosphingolipids in endocytic uptake processes of renal proximal tubules, which may be relevant for acute kidney failure caused by nephrotoxic agents. Intra-vital microscopy points to dynamic differences in albumin reuptake between wild type and Gb3 synthase-deficient mice during the early phase of FITC-albumin exposure. The molecular mechanisms remain to be solved. To elucidate the function of glycosphingolipids in tubular reuptake *in vitro*, we generated knockout cell lines from the human proximal tubular epithelial cell line HK-2. We were able to show a reduction in albumin uptake in Gb3 synthase deficient cells. Furthermore, we generated cell lines that will allow to identify specific

interactions between sphingolipids and proteins by proximity crosslinking. To target these dynamic mechanisms *in vivo*, we are establishing reporter mice for defined endocytic uptake mechanisms. Unravelling the mechanism of Gb3-dependent reuptake may help to identify targets to prevent certain cases of nephrotoxicity.

[1] Ivan Morace, Robert Pilz, Giuseppina Federico, Richard Jennemann, Damir Krunic, Viola Nordström, Johanna von Gerichten, Christian Marsching, Ina Maria Schießl, Johannes Müthing, Christian Wunder, Ludger Johannes, Roger Sandhoff and Hermann-Josef Gröne. Renal globotriaosylceramide facilitates tubular albumin absorption and its inhibition protects against acute kidney injury (in revision).

Towards a biopsy at the nanoscale: Novel approaches and recent advances in cryo-electron tomography

Presenting author: **Jürgen Plitzko**

Cryo-focused ion beam (cryo-FIB) milling of frozen hydrated cells to produce thin lamellas in combination with cryo-electron tomography (cryo-ET) has led to unprecedented insights into the cell interior. This method opens the door to native structures deep inside the cells and makes structural investigations of macromolecules *in situ* possible. Various implementations of cryo-FIB milling for cells deposited or grown directly on EM grids have been realized, and some preliminary results suggest that this approach can be extended to high-pressure frozen tissue. However, a small volume of interest must be extracted from a bulky sample, i.e. a process analogous to a clinical biopsy, which can be performed under cryogenic conditions.

While the preparation process must be specially tailored to the peculiar properties of the sample, it is also necessary to adapt the tomographic recording and analysis process to both the sample and the respective research question. Unfortunately, there is no universal recipe, neither for sample preparation nor for data acquisition, and neither for unlocking and decoding the information content of tomograms, that is suitable for each individual sample.

In this lecture, we will present our recent work in the field of cryo-ET and *in situ* structural biology, highlighting technological developments and their potential, and offering a perspective to obtain "anatomical" details at the molecular level from larger cells or tissues - towards a biopsy at the nanoscale.

Integrative structural analysis of the HOPS complex using X-ray crystallography and single-particle electron microscopy

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Eukaryotic cells rely on vesicle trafficking for growth, differentiation, signaling, and many other crucial cellular functions. Vesicles deliver their cargoes by fusing with the membranes of other vesicles, cellular organelles or the plasma membrane. The driving force behind these fusion reactions is the assembly of

SNARE proteins anchored in the two membranes into membrane-bridging complexes. The specificity and efficiency of these fusion reactions is established by the intricate interplay of SNAREs, Rab GTPases, Sec1/Munc18 (SM) proteins and multisubunit tethering complexes (MTCs). MTCs in particular appear to be the major orchestrators of vesicle tethering and fusion, as they interact with SNAREs and most of the other trafficking co-factors, but the underlying molecular mechanisms are yet to be unraveled.

One of the best-characterized MTCs, the HOPS (homotypic fusion and vacuolar protein sorting) complex is essential for the fusion of late endosomes, lysosomes and autophagosomes. HOPS is a ~660 kDa, hexameric complex and contains the SM protein Vps33 as an integral subunit. We are using an integrative approach combining X-ray crystallography with single particle negative stain- and cryo-electron microscopy to determine a high-resolution structure of the entire HOPS complex. Elucidating the architecture of the HOPS complex, and deciphering its interactions with the other proteins of the vesicle trafficking machinery will shed light on how MTCs orchestrate the correct regulation and high specificity of membrane tethering and fusion.

Mechanism of loading and translocation of type VI secretion system effector Tse6

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The type VI secretion system (T6SS) is a key mediator of microbial competition. Functioning as a syringe-like injection apparatus it punctures the membranes of contacting cells, thereby delivering a multitude of effector proteins. While the mode-of-action for several of these effectors has been unraveled in recent years, to date little is known about the mechanisms underlying their loading onto the apparatus or their subsequent translocation into target cells. Here, using cryo-electron microscopy, we analyzed the structures of the *P. aeruginosa*effector Tse6 loaded onto the T6SS spike protein VgrG1 in both its soluble form and embedded in lipid nanodiscs. In the absence of membranes, two copies of the dimeric chaperone EagT6 are required for Tse6 stability, accommodating the two transmembrane segments of Tse6 in their hydrophobic cavities. EagT6 is not directly involved in Tse6 delivery but crucial for its loading onto VgrG1. Furthermore, we demonstrated the ability of Tse6 to spontaneously enter membranes and translocate its enzymatic $NAD(P)^+$glycohydrolase domain across lipid bilayers, indicating that the T6SS does not require puncturing of the target cell inner membrane by VgrG1. Collectively, our findings suggest a refined model for the loading of Tse6 onto the T6SS and its subsequent delivery into recipient cells, shedding light on the elusive role of chaperones in the process and thus advancing our understanding of this intriguing molecular machine.

Novel Tools towards Magnetic Guidance of Neurite Growth in Dopaminergic Cells: Cytoplasmic Capturing and Guidance of Magnetic Nanoparticles Functionalized with Neurite-Promoting RAS Proteins

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Author(s):
Fabian Raudzus, Hendrik Schöneborn, Emilie Secret, Rolf Heumann

Parkinson's disease (PD) is a neurodegenerative disorder caused by the progressive loss of dopaminergic neurons in the *substantia nigra*. Up to now, no cure for PD is available and treatments such as deep brain stimulation (DBS) or pharmacological medication like levodopa (L-DOPA) only alleviate typical symptoms such as tremor, rigidity, and akinesia. Novel strategies such as the autologous cell replacement seem to be promising to treat Morbus Parkinson (Heumann et al. 2014). However, the fundamental challenge is the directed fiber outgrowth of grafted cells from the *substantia nigra* towards the *striatal* target site. This proper re-innervation is a prerequisite to restore the physiological condition.

Our aim is to remote control local RAS pathway activation by magnetic nanoparticles (MNPs). Therefore, we use the HaloTag® system to biofunctionalized these MNPs with RAS GTPase or its guanine nucleotide exchange factor SOS1. The *in vitro* characterization of biofunctionalized MNPs is determined by multi-angle light scattering (MALS). Moreover, we investigated the biological activity of the HaloTag® fusion proteins. Furthermore, the cytoplasmic capturing and asymmetric membrane alignment, as well as the guidance of MNPs into neurite extensions, are shown by time-lapse fluorescence microscopy. After HaloTag® modification, nanoparticles are delivered into the cytoplasm of target cells thereby aiming to act as intracellular signaling platforms. These tools are thought to contribute in future experiments to the magnetic guidance of neurite growth in induced human dopaminergic neurons.

Heumann, R. et al. (2014). Dyskinesia in Parkinson's disease: mechanisms and current non-pharmacological interventions. Journal of Neurochemistry, DOI: 10.1111/jnc.12751

Towards the Amyloid Fibril Structure of PI3K-SH3 by use of Cryo-Electron Microscopy

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Src homology 3 (SH3) domains mediate protein-protein interactions in numerous signaling pathways. In its native fold, SH3 domains have been used as model systems for protein folding studies for decades. The SH3 domain of phosphatidylinositol-3-kinase (PI3K-SH3) was among the first proteins discovered to form *in-vitro* amyloid fibrils, while not being associated to any known human disease.

1999, first cryo-electron microscopy (cryo-EM) data of the amyloid fibril of PI3K-SH3 have been published. Now, exactly 20 years later, we present the first atomic model of the PI3K-SH3 amyloid fibril with a resolution determined to 3.4 Å. The fibril is composed of two intertwined protofilaments with a 2_1-screw symmetry and an interestingly large interface consisting of mainly two, small hydrophobic clusters. The model comprises residues 1-77 out of 86 amino acids in total, with the missing C-terminal residues clearly indicating a high flexibility. Complementing information on the fibril formation through point mutation experiments reveal, that minor sequence changes may prohibit fibril formation

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Structures of a Tc holotoxin reveal the mechanism of activation and translocation

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Bacterial toxins play important roles in infectious diseases and can serve as alternative to insecticides. The heterotrimeric Toxin Complex (Tc) of the insect-pathogen Photorhabdus luminescens is a modular toxin system that is composed of a 1.4 MDa, pentameric membrane translocator (TcA) and a 270 kDa cocoon encapsulating the toxic enzyme, an ADP-ribosyltransferase (TcB and TcC). Upon toxin activation, TcA binds to the target cell, and a pH shift triggers the conformational transition from the soluble pre-pore form to the membrane-embedded pore. Subsequently the ADP-ribosyltransferase is translocated through the channels of TcB and TcA and is released into the cytoplasm.

Here we report the overall structures of the 1.7 MDa holotoxin in the pre-pore form and in the pore form in lipid nanodiscs, obtained by electron cryo-microscopy. We show that binding of the TcB-TcC cocoon to the TcA channel triggers the opening of the cocoon and the specific loading of the toxic enzyme into the channel. The presence of the toxic enzyme inside the cocoon is essential for holotoxin formation. The enzyme passes through a narrow constriction site with alternating negative charges and hydrophobic stretches inside the cocoon, acting as an extruder that releases the unfolded protein with its C-terminus first into the translocation channel.

Fluorescent covalently labelled calcium phosphate nanoparticles by click chemistry for cellular imaging

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Calcium phosphate based nanoparticles are frequently used in biological applications such as carrier for biomolecules, drugs, and applied, e.g., for transfection and siRNA gene silencing in vitro and in

vivo. Our group has developed methodologies for the synthesis and modification of silica-terminated calcium phosphate nanoparticles for different applications[1-3].

Highly fluorescent labelled calcium phosphate nanoparticles were prepared with different dyes (i.e. FAM, TAMRA, AF488, Cy5, aromatic thioether with aggregation-induced emission (AIE) properties) using click chemistry reactions such as the copper-catalyzed azide-alkyne cycloaddition (CuAAC) and the strain-promoted azide-alkyne cycloaddition (SPAAC)[3]. The obtained nanoparticles were fully characterized and had an average solid core diameter of around 90 nm (from SEM) and a hydrodynamic diameter of 200 nm (from DLS). Furthermore, the nanoparticles were studied before and after cellular uptake by Hela cell line using different fluorescence and super-resolution microscopy techniques.

This approach allows to have a stable covalent bond with the molecules on the calcium phosphate nanoparticles surface and other alkyl-terminate molecules, like modified proteins or antibodies, can potentially be coupled to the nanoparticles increasing the possibilities for cellular targeting and imaging. Moreover, inside the nanoparticle core is possible to add an additional cargo for delivery making the system fully versatile.

The Nanoscale Organization of the B Cell Membrane

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The B cell antigen receptor (BCR) is central to the specific activation of cognate B lymphocytes during an infection or a successful vaccination leading to antibody production and humoral immunity. The signaling behavior of the BCR cannot be fully understood without a better knowledge of the nanoscale organization of the B cell membrane. Mounting data from newer studies demonstrate that the plasma membrane (PM) of B cells show a high degree of compartmentalization of its surface molecules. Using super-resolution techniques of dSTORM, TEM and a Fab fragment-based proximity ligation assay (Fab-PLA) in fixed samples, our lab has shown that two isotypes of the BCR expressed on mature B cells, namely IgM-BCR and IgD-BCR reside in different protein islands with distinct protein and lipid composition. By using a fast and high-resolution confocal laser scanning microscopy technique (Airyscan, Zeiss), we are now conducting 3D imaging studies on live B lymphocytes with time axis to visualize the dynamics of these protein islands on the PM and in the secretory and endo-lysosomal networks. 3D imaging in B cells is crucial to obtain information about the distinct localization of PM molecules due to the B cells' highly curved spherical shape. By using single molecule TIRF microscopy, we have found that the attachment of B cells to a coverslip can dramatically change the diffusion behavior of both IgM-BCR and IgD-BCR and may lead to aberrant conclusions about localization. By combining high-resolution live 3D imaging of B lymphocytes and the CRISPR/Cas9 gene editing technology, we are searching for elements for the organizational stability of the BCR protein islands and studying the possible contribution of endo- and exocytic events in the formation of this distinct organization.

Protein structure and dynamics using X-ray free-electron lasers

Presenting author: **Ilme Schlichting**

Abstract not submitted

Interrogating the Cell Biology of Infection and Immunity with Camelid Nanobodies

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Distinct cytosolic sensors nucleate the formation of inflammasomes in response to signs of infection or danger to coordinate the appropriate inflammatory response. Yet, the molecular details of inflammasome signalling are only poorly understood, mostly due to the lack of appropriate tools. We investigate inflammasome activation and its downstream consequences using alpaca variable domains of heavy chain-only antibodies (VHHs), also known as nanobodies. These 15 kDa single domain antibody fragments can a) be expressed in the cytosol of living cells to visualize and interfere with protein function, and b) be recombinantly produced in bacteria and modified for use in advanced microscopy methods, including proximity ligation assays and super resolution microscopy.

We immunized alpacas with different inflammasome components and identified nanobodies against diverse inflammasome components by phage display. Using VHH_{ASC}, a nanobody against the CARD domain of the inflammasome sensor ASC, we illustrate the strength of these novel tools: We solved the structure of the nanobody in complex with its target, and used VHH_{ASC} to visualize and purify ASC from the relevant cell types. We further expressed the nanobody in human macrophages and specifically interfered with inflammasome function in living cells and in vitro. We gain mechanistic insights into the assembly of inflammasomes by stabilizing and visualizing an informative intermediate of inflammasome assembly. Using nanobodies against other inflammasome components, we can artificially activate inflammasomes, or inhibit cell death by pyroptosis.

We expect that these novel tools will allow us to subject the intricate details of inflammatory signaling cascades to detailed microscopic analysis in cell lines and primary cells.

How basic research in rodents contributes to the molecular understanding of complex human diseases. – The Cav2.3 / R-type voltage-gated Ca2+ channel.

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In the human gene of the pharmacoresistant $Ca_v2.3$/R-type calcium channel *de novo* pathogenic mutations were detected in a group of 30 individuals with developmental and epileptic encephalopathies (1). A short historical overview for the performed basic research in rodents will be provided, and the reasons for an improved understanding of the published human mutations will be described. Interestingly, most of the channel mutations cluster within the cytoplasmic ends of the four S6 transmembrane segments, which constitute part of the $Ca_v2.3$-channel activation gate.

For the mutants identified in domain II of human $Ca_v2.3$, recombinant studies have shown that the distal part of $Ca_v2.3$ is important for the stability of the open state of $Ca_v2.3$ (2). Further, the first evidence for a strong electromechanical coupling between S4-S5 and the S6 of domain II came from a double mutant cycle analysis (3).

The summary of recent work in rodents (rats and mice) focused on the mechanisms of hyperexcitation caused by the KA-mediated activation of $Ca_v2.3$ illustrates how understanding of a complex human disease like developmental and epileptic encephalopathy (DEE) is improved by basic research data. However, future investigations must follow, which should provide therapeutic strategies for prevention of the observed fatal developmental disturbances. One promising step will be the extremely high sensitivity of $Ca_v2.3$ towards bioavailable trace metal cations like Zn^{2+} and Cu^{2+}(4).

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Fibril structure and interface polymorphism of amyloid-ß(1-42)

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Amyloids are associated with many diseases. Fibrillar aggregates of the amyloid-β protein (Aβ) are the main component of the senile plaques found in brains of Alzheimer's disease patients. We present the structure of an Aβ(1-42) fibril determined by cryo-EM to a resolution of 4.0 Å. The presented polymorph of the fibril consists of two intertwined protofilaments in which individual subunits form a 'LS'-shaped topology. It is known that the surface of the Aβ fibrils catalyzes the formation of new oligomers and fibrils. This secondary nucleation process suggests the existence of alternative interfaces of Aβ with the fibril. We further present the structures of two other polymorphs which differ in the interfaces between the LS-shaped subunits. The observed interface polymorphism might provide insight into possible secondary nucleation mechanisms.

Long-term single-cell quantification: New tools for old questions

Presenting author: **Timm Schroeder**

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Despite intensive research, surprisingly many long-standing questions in cell research remain disputed. One major reason is the fact that we usually analyze only populations of cells - rather than individual cells – and at very few time points of an experiment – rather than continuously. We therefore develop imaging systems and software to image, segment and track cells long-term, and to quantify e.g. divisional history, position, interaction, and protein expression or activity of all individual cells over many generations. Dedicated software, machine learning and computational modeling enable data acquisition, curation and analysis. Custom-made microfluidics devices improve cell observation, dynamic manipulation and molecular analysis. The resulting continuous single-cell data is used for analyzing the dynamics, interplay and functions of signaling pathway and transcription factor networks in controlling hematopoietic, pluripotent, skeletal and neural stem cell fate decisions.

After the revolution (in resolution): Where do we go from here?

Presenting author: **Philipp Selenko**

Recent breakthroughs in optical and electron microscopy have changed the fields of Cellular and Structural Biology in a most profound manner. Ever more detailed information about the inner workings of cells is becoming available, revealing stunning new insights into cellular landscapes and their biological activities, at unprecedented levels of resolution. Besides these advancements in imaging modalities, complementary *in situ* methods are beginning to emerge as powerful tools in modern Cellular Structural Biology approaches. Here, I discuss how recent developments in in-cell NMR, EPR and single-molecule FRET spectroscopy contribute to our understanding of basic biological processes in live cells. Specifically, I outline how these techniques provide time-resolved atomic-resolution information about intracellular protein structures and functions, which cannot be obtained with any other method at this time.

Studying BOK Protein on The Single-Molecule Level

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Apoptosis is a crucial process for differentiation, development and homeostasis. Its initiation follows either an intrinsic or extrinsic pathway. The BCL-2 family of proteins are important mediators of the intrinsic pathway of apoptosis by triggering mitochondrial outer membrane (MOM) permeabilization. Dysregulation of this sophisticated protein interaction network has been implicated in various diseases, including cancer. The BCL-2 ovarian killer (BOK) protein has a high sequence similarity to both pro-apoptotic effectors BAK and BAX, but with less understood role in apoptosis regulation. In contrast to other BCL-2 proteins which are mainly located on the MOM, BOK is predominantly resident on the endoplasmic reticulum membrane. Recently, BOK core structure has been solved by X-ray

crystallography and NMR, both reflecting to the protein structure in aqueous environments. Here, we have examined the organization of BOK in membrane environments using single-molecule brightness experiments. To enable this method, expression and purification of a truncated form of BOK lacking the C-terminal tail were optimized to yield the protein in a monomeric form with high purity. Different model membrane systems were formulated and used to assess the function and binding preference of BOK. Sortase-mediated labelling was performed for the coupling of the fluorescent dye (Atto488) to the protein N-terminus with very high labelling efficiency and controlled stoichiometry. The results will pave the way to harvest structural information on BOK at different levels of detail and in various environments.

The distinct properties of thylakoid membranes control IM30-mediated membrane fusion

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The thylakoid membranes (TMs) of chloroplasts and cyanobacteria are essential for oxygenic photosynthesis. Nevertheless, not much is known about the formation and maintenance of this special membrane system. The *inner membrane-associated protein of 30 kDa* (IM30/Vipp1) seems to be crucially involved in the dynamics of the TMs. However, the exact physiological function od this protein is still unclear, although several potential functions were postulated within the last years. Our group has shown that *in vitro* upon membrane binding, IM30 of the cyanobacterium *Synechocystis* sp. PCC 6803 destabilizes the membrane structure and mediates fusion of liposomes in presence of $MgCl_2$. Such membrane fusion processes are likely crucial for inner membrane dynamics *in vivo*.

We investigate the membrane interactions of IM30 with different *in vitro* methods including a FRET-based liposome fusion assay, electron- and fluorescence microscopy. As TMs have a very special lipid composition and architecture, we have now analyzed which membrane properties are crucial for the fusogenic activity of IM30. Our observations suggest that the chloroplast and cyanobacteria specific, non-bilayer-forming galactolipid MGDG, as well as negatively charged lipids are essential for IM30-mediated membrane fusion. Furthermore, also the membrane curvature clearly has an impact on the fusion rates, as fusion is enhanced for highly curved liposomes. This indicates that fusion might happen at highly curved TM margins *in vivo*. Interestingly, this is exactly where IM30 has been found to localize in high concentrations in *Synechocystis*. Thus, we propose a special activity of IM30 at TM boundaries in chloroplasts and cyanobacteria.

Iron-sequestering Encapsulins as multi-plexed gene reporters for Electron Microscopy

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Encapsulins are a recently discovered class of prokaryotic semi-permeable spherical nano-compartments that are implicated in oxidative stress response and iron-biomineralization(McHugh et al. 2014; He et al. 2016). We have recently established that the ~32 nm T=3 encapsulin of M.xanthus (MxEnc) can be heterologously expressed in mammalian cells and due to its geometrical features and electron-dense iron core serve as a fully-genetic cell identity marker for Electron Microscopy (EM)(Sigmund et al. 2018). Herein, we introduce the encapsulin system of Q.thermotolerans (QtEnc) and show via cryoEM reconstruction that it forms significantly larger icosahedral T=4 nano-spheres of ~44 nm diameter. Besides its larger size, we show that it bio-mineralizes iron more efficiently than MxEnc via its ferroxidase activity-possessing IMEF cargo protein. Furthermore, we imaged HEK293T cells expressing the different iron-mineralizing encapsulin systems using conventional TEM from plastic sections and show that Machine-learning based image classification can robustly differentiate between the two encapsulin classes. By attaching a C-terminal farnesylation-signal, we demonstrate that encapsulins can also be directed to specific subcellular locations as shown on TEM images(Sigmund et al. 2019). In conclusion, we enable multiplexed EM gene reporter imaging in mammalian cells by introducing an additional encapsulin system with distinct geometrical features. For the future, we envision an even further expanded genetically-encodable 'color-palette' of 'EMcapsulins' of different sizes that will be helpful in EM-driven micro-connectomics research.

Structural Basis of co-translational protein folding

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During protein synthesis at the ribosome numerous factors act early on the nascent polypeptide chain. These can be grouped into three major classes – chaperones that assist in folding, enzymes that modify the nascent chain and targeting factors that assist in protein localization. As all of them need access to the nascent polypeptide chain, they utilize partially overlapping binding sites at the ribosomal tunnel exit, but their interplay is poorly understood. Our data provide the structural framework for interactions of co-translational factors at the ribosomal tunnel exit. In yeast, the canonical Hsp70 protein Ssb acts together with the ribosome associated complex (RAC), which consists of the inactive Hsp70 protein Ssz and the Hsp40 protein Zuotin. Together, they form a unique chaperone triad at the ribosome. Structure determination of Ssb and RAC together with ribosome binding studies provide detailed insights into the interplay of this chaperone system, which evolved to link translation and protein folding.

Membrane binding mechanism of the large GTPase hGBP 1

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Human guanylate binding proteins (hGBPs) belong to the dynamin superfamily of large GTPases and they are upregulated in cells as an immune response against microbes and viruses. Three of the seven isoforms can be isoprenylated, which allows the hGBPs not only to be localized in the cytosol, but also to attach to organelle and plasma membranes as well as to pathogen membranes.

To gain a better understanding of the cellular functions of hGBP 1, we try to unscramble the nucleotide-induced homo-interaction mechanism of farnesylated hGBP 1 (hGBP 1_{fn}). On the one hand we consider the protein interaction that leads to binding to the membrane and on the other hand the interaction that results from binding to the membrane. Therefore, we observe the binding of hGBP 1_{fn} to artificial membranes, using giant unilamellar vesicles (GUVs), with the help of confocal fluorescence microscopy. Several interaction partners are offered to hGBP 1_{fn} full length to be guided to the membrane in a nucleotide dependent manner. These interaction partners have in common, that they can not interact with the membrane on their own.

We suggest, that dimer formation is crucial for membrane binding, implying that also non-farnesylated protein can be guided to the membrane, while the interaction between the hGBPs seems to be more complex when already bound to the membrane.

ATG-18 and EPG-6 are required for autophagy and play opposing roles in lifespan control of Caenorhabditis elegans

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During macroautophagy, the human WIPI (WD-repeat protein interacting with phosphoinositides) proteins (WIPI1-4) function as phosphatidylinositol 3-phosphate (PI3P) effectors at the nascent autophagosome. Likewise, the two WIPI homologues in *Caenorhabditis elegans*, ATG-18 and EPG-6, play important roles in autophagy whereby ATG-18 is considered to act upstream of EPG-6 at the onset of autophagy. Due to its essential role in autophagy, ATG-18 was found to be also essential for lifespan extension in *Caenorhabditis elegans*, however this has not as yet been addressed with regard to EPG-6. Here, we wished to address this point and generated mutant strains that express the autophagy marker GFP::LGG-1 (GFP-LC3 in mammals) and harbour functional deletions of either atg-18 (*atg18(gk378)*), epg-6 (epg-6(bp242)), or both (*atg-18(gk378);epg-6(bp242)*). Using quantitative fluorescence microscopy, western blotting and lifespan assessments we provide evidence that in the absence of either ATG-18 or EPG-6 autophagy was impaired. Only atg-18 mutant animals showed a short-lived phenotype whereas lifespan significantly increased in epg-6 mutant animals. We speculate

that the long-lived phenotype of epg-6 mutant animals points towards an autophagy-independent function of EPG-6 in lifespan control that warrants further mechanistic investigations in future studies.

Structural analysis of membrane-bound dynamin-like EHD-ATPases by X-ray crystallography and Subtomogram Averaging

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Eps15 homology domain containing proteins (EHDs) comprise a family of ubiquitously expressed dynamin-related ATPases which regulate diversified membrane trafficking and membrane remodeling pathways. In an ATP-dependent manner EHDs assemble on membranes to form oligomeric scaffolds that remodel the underlaying membrane and stabilize membrane curvature. Despite recent progress in the field[(1)], the lack of high-resolution structural information of membrane-bound EHDs precludes a mechanistical understanding of the underlying molecular mechanisms of membrane recruitment and remodeling. To understand the principles of assembly, function and regulation of these ATPases we applied an integrative approach that combines structural studies with biochemical and cell-based assays. Here we present our progress in the analysis of EHD proteins in the oligomerized form, using a combination of X-ray crystallography, cryo-electron tomography and Subtomogram Averaging to reveal the conformational changes associated with membrane interactions of EHDs.

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High-resolution structure determination of dynamic macromolecular complexes

Presenting author: **Holger Stark**

Single particle cryo electron microscopy (cryo-EM) has developed into a powerful technique to determine 3D structures of large macromolecular complexes. Due to improvements in instrumentation and computational image analysis, the number of high-resolution structures is steadily increasing. The method cannot only be used to determine high-resolution structures but also to study the dynamic behavior of macromolecular complexes and thus represents a very complementary method to X-ray crystallography. We have recently determined the structure of human proteasomes and their inhibition by anti-cancer drugs using X-ray crystallography to visualize the chemistry of inhibition at unprecedented resolution of 1.8 Å. By cryo-EM we were able to visualize the long-range allosteric conformational changes induced by the drug binding and visualized the effects of drug binding in terms of restrictions in the free-energy landscape of the human 26S proteasome. More examples of cryo-EM studies of dynamic processes in large macromolecular complexes will be presented at the conference.

Photo-switching in optoacoustics

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Reversibly switchable fluorescent proteins (rsFPs) have had a revolutionizing effect on life science imaging due to their contribution to subdiffraction-resolution optical microscopy. RsFPs might have yet another lasting impact on Photo- or Optoacoustic imaging (OA). OA combines optical contrast with ultrasound resolution enabling high-resolution real time in vivo imaging well-beyond the 1 mm penetration depth typical of microscopy methods. However, the method suffers from low availability of dedicated transgene labels. Moreover, the existing labels are notoriously bad to detect among the vast amounts of other absorbing material prevalent in tissue like blood or lipids. Now, several studies could show that modulation of the absorption bands in rsFPs (photochromism) can be exploited to delineate the labels from strong background absorbers rendering them virtually invisible (locked-in detection). In our work we show i) how the experimental factors central to OA imaging influence the photophysics of the different types of rsFPs. ii) We explore the binding of the biliverdin chromophore – essential for mammalian imaging and structurally elucidate the link between oligomerization and chromophorylation. iii) Finally, we develop and apply dedicated reversibly switchable proteins for OA (rsOAPs) allowing for longitudinal in vivo tomography studies of developmental processes, immune responses and tumor biology.

Cryo-EM structures of KdpFABC reveal K(+) transport mechanism via two inter-subunit half-channels

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KdpFABC is a key player in prokaryotic K^+ homeostasis. When faced with externally low micromolar K^+ concentrations the constituvely expressed K^+ uptake systems KtrAB and TrkAH fail to maintain the intracellular K^+ concentration [1]. Instead, KdpFABC is produced, which actively transports K^+ into the cells. KdpFABC consists of four subunits and is often referred to as P-type ATPase, since subunit KdpB belongs to this superfamily. However, KdpB is believed to solely hydrolyse ATP, while K^+ translocation is accomplished by the channel-like subunit KdpA. The periplasmatically oriented KdpC and the lipid-like single spanner KdpF complete the unique complex [2]. Till now, the coupling mechanism between the spatially separated energizing and transporting units remained elusive.

Here, we present two cryo-EM structures of the 157 kDa, asymmetric KdpFABC complex at 3.7 Å and 4.0 Å resolution in an E1 and an E2 state, respectively [3]. Unexpectedly, the structures disclose a new translocation pathway through two half-channels along KdpA and KdpB, uniting the alternating-access mechanism of actively pumping P-type ATPases with the high affinity and selectivity of K^+ channels.

This way, KdpFABC functions as a true chimera, synergizing the best features of otherwise separately evolved transport mechanisms.

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Near-infrared STED nanoscopy with an engineered bacterial phytochrome

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The near-infrared (NIR) optical window between 650 nm and 900 nm is preferable for live-cell deep-tissue fluorescence imaging due to reduced phototoxicity and increased light penetration depth compared to conventionally employed UV/VIS light. Unfortunately, no fluorescent protein (FP) with an excitation maximum in the NIR window could be engineered from the GFP family. Bacteriophytochromes are new promising templates for the directed evolution towards bright, stable and monomeric near-infrared fluorescent protein labels.

Here, we present the near infrared fluorescent protein SNIFP, a bright and photostable bacteriophytochrome, and demonstrate its use as a fusion tag in live-cell confocal microscopy and STED nanoscopy.

Cryo-EM and drug discovery

Presenting author: **Sriram Subramaniam**

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Recent breakthroughs in the field of cryo-electron microcopy (cryo-EM) provide new prospects for determination of the structures of a variety of medically important macromolecular assemblies. The prospect that the determination of protein structures at atomic resolution will no longer be limited by size, or by the need for crystallization represents a significant and exciting horizon in structural biology. In addition, advances in technologies for imaging viruses, cells and tissues in 3D have created new avenues for structural imaging in 3D and for integrating information across length scales. In my talk, I will discuss the broader context of the development of cryo-EM methods for studying large biological assemblies, and provide an overview of current progress and future directions as they relate to the development of improved therapeutic agents.

Imaging nanoscale aggregation of proteins ex vivo using the contrast in Förster resonance energy transfer obtained from 2D polarization fluorescence imaging (2D POLIM)

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Author(s):
Daniela Täuber, Juanzi Shi, Rafael Camacho, Ivan G. Scheblykin

Förster resonance energy transfer (FRET) is a well-established nanoruler suited to discriminate small aggregates of fluorescent molecules from just high concentration. Contrary to spectrally resolved two-color-FRET, polarization resolved fluorescence microscopy allows to determine homo-FRET between similar fluorophores. Conventional fluorescence anisotropy is restricted to isotropic samples, otherwise, the results depend on the choice of the lab frame. 2D POLIM provides imaging contrasts in mean fluorescence intensity, and polarization properties of the sample, which are fluorescence anisotropy, modulation depths of polarization in excitation and emission, and main polarization angles in excitation and emission. A further imaging contrast quantifies the amount of homo-FRET between the labels, which provides information on the formation of nanoscale aggregation.

2D POLIM was recently applied to study early protein aggregation of GFP-labeled human alpha-synuclein in models of Parkinson's disease ex vivo.[1] Current investigation of f-actin aggregation in liver tissue using 2D POLIM shows promising first results.

[1] Camacho et al. 2D Polarization Imaging as a Low-Cost Fluorescence Method to Detect alpha-Synuclein Aggregation Ex Vivo in Models of Parkinson's Disease. Commun. Biol. 2018, 1, 157.

Nanoscale chemical imaging of cell surface and core-shell nanoparticles using infrared excitation and detection by force microscopy

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Combining excitation in the mid infrared by quantum cascade lasers with detection using a conductive AFM tip allows for chemical imaging with nanoscale resolution. Imaging contrasts of the sample can be obtained at selected excitation wavenumbers or via hyperspectral imaging. This allows for investigation of the chemical composition of surfaces on areas of up to 100 µm while the spatial resolution can be scaled down to a few nanometers.
First results from investigation of multicore magnetic nanoparticles with polydehydroalanine (PDha) coating and outer perylene adsorbed to this shell allowed for discrimination of the perylene shell from the PDha coating. Such nanoparticles are of interest for biomedical applications. The new label-free method is independent of stray light and a promising tool for complementing established

spectroscopy techniques for investigation of cell surfaces and tissue materials. Currently its performance in comparison to IR- and Ramanspectroscopy is investigated.

Nuclear pores as universal reference standards for quantitative microscopy

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Recent advances in superresolution microscopy now allow us to address structural questions in cell biology with optical methods. A quantitative interpretation however is often limited by sub-optimal performance and calibration of the microscope, undetermined performance of the fluorescence label and imaging conditions, unknown labeling efficiencies and systematic errors in counting protein numbers.<br style="font-family:Helvetica;font-size:12px;" /><br style="font-family:Helvetica;font-size:12px;" />Here we show that the use of reference standards can overcome these limitations and greatly improve quantitative microscopy. To this end we exploit the precise 3D arrangement and stoichiometry of proteins in the nuclear pore complex.<br style="font-family:Helvetica;font-size:12px;" /><br style="font-family:Helvetica;font-size:12px;" />We present a set of genome edited cell lines in which we endogenously labeled the nucleoporin Nup96 with eGFP, SNAP- or HALO-Tag or the photoconvertible fluorescent protein mMaple. We demonstrate their use as a) simple and robust resolution standards for calibration and quality control, b) accurate assays to quantify absolute labeling efficiencies in superresolution microscopy and c) precise counting reference standards for absolute stoichiometry measurements.<br style="font-family:Helvetica;font-size:12px;" /><br style="font-family:Helvetica;font-size:12px;" />As a resource shared with the community, these cell lines will enable many groups to assess the quality of their microscopes and labels and to perform quantitative, absolute measurements.

3D electron microscopy and confocal live imaging reveal the importance of axon-glia adhesion during CNS myelination

Presenting author: **Sebastian Timmler**

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Central nervous system myelin is a multi-layered membrane stack enabling fast nerve conduction and providing trophic support to ensheathed axons. A cellular model of myelin generation by oligodendrocytes was proposed, but the molecular mechanisms of myelination such as initiation, lateral growth and termination stay elusive. Using confocal live-imaging in zebrafish larvae and 3D electron microscopy techniques of mouse tissue, we show that adhesion molecules of the paranodal

axo-glial junction and the internodal segment together regulate axonal targeting and myelin wrapping. Absence of these adhesion systems results in myelin mistargeting on top of already established myelin segments, overgrowth of nodes of Ranvier and enwrapping of neuronal cell bodies. Additionally, the myelin leading edge can detach from the axon and invade previously formed layers of the same sheath. We show how two adhesive systems synergistically guide axonal ensheathment and myelin wrapping, and provide a mechanistic insight of how myelin generation is orchestrated at the molecular level.

How peptide-loading complex affects MHC class I biology

Presenting author: **Simon Trowitzsch**

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Identifying and eliminating infected or malignantly transformed cells are fundamental tasks of the adaptive immune system. For immune surveillance, the cell's metastable proteome is displayed as short peptides on MHC-I molecules to cytotoxic T-lymphocytes. Interestingly, viruses have evolved immune evasion strategies, which target the antigen-presentation pathway. Our knowledge about the track from the proteome to presentation of peptides has greatly expanded in recent years and has lead to a comprehensive understanding of antigen presentation and how viruses escape immune surveillance. In the ER membrane the ABC transporter TAP assembles the peptide-loading complex (PLC), which serves as a central checkpoint during peptide/MHC-I formation. Due to the intrinsically dynamic nature and the heterogeneous composition of the complex its molecular organization remained largely unknown. We have used cryo-EM to determine the structure of native PLC isolated from Burkitt's lymphoma cells using an engineered viral inhibitor. We identified two editing modules composed of tapasin, calreticulin, ERp57, and MHC-I centered around TAP. Multivalent chaperone networks ensure formation of thermodynamically stable peptide/MHC-I complexes. Calreticulin anchors to tapasin and ERp57 and holds MHC-I at the PLC in a glycan-dependent manner. This arrangement allows tapasin to stabilize MHC-I molecules in a peptide-receptive state while concomitantly facilitating peptide editing. The overall architecture suggests that peptides accumulate in a cavity and channel through lateral windows towards MHC-I. Structures of PLC at distinct assembly states provide insights into MHC-I quality control and unveil the molecular details underlying the onset of an adaptive immune response.

Analyzing transmembrane ribitol flux mediated by GlpF or Aqp3 using RDH-loaded proteoliposomes

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Aquaglyceroporins, such as the human Aqp3 or GlpF of *E. coli*, facilitate the flux of water, glycerol and other small molecules into the cytosol. The active form of these membrane proteins is a homotetramer, albeit a water/glycerol channel is located in each monomer. Both, GlpF and Aqp3 have been shown to transport diverse polyalcohols *in vitro*, including ribitol.

We aim to generate a coupled system to analyze the ribitol flux across membranes, mediated by GlpF and Aqp3, *in vitro*. The addition of ribitol causes water efflux from proteoliposomes, due to osmotic effects, leading to shrinkage of the liposomes. Thereupon, passive transport of ribitol along the concentration gradient mediated by GlpF or Aqp3 results in re-swelling of the proteoliposomes. The changes in liposome sizes accompanying this process can be detected via light scattering, which is frequently used to study GlpF- or Aqp3-mediated ribitol flux.

Using ribitol dehydrogenase (RDH) from *Rhodobacter sphaeroides* allows measuring the transport velocity of GlpF and Aqp3 via NADH absorbance more precisely, as RDH is a short chain dehydrogenase/reductase that oxidizes NAD^+ to reduce ribitol to D-ribulose. For this coupled transport activity assay, GlpF or Aqp3 has to be reconstituted in proteoliposomes containing RDH and NAD^+. The turnover of NAD^+ to NADH, which is directly proportional to the channel activity of GlpF and Aqp3, is then determined by measuring absorbance changes at 340 nm.

A monoclonal antibody against bacterially expressed MPV17 sequences does not stain Mitochondria and lacks staining in Human Mpv17 knock out cells; in support of nucleotide bypass therapy for patients with Mpv17 deficiency disease

Presenting author: **Hans Weiher**

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Mitochondrial DNA (mtDNA) maintenance defects (MDMD) are a group of diseases caused by deficiency of proteins involved in mtDNA synthesis, mitochondrial nucleotide supply, or mitochondrial dynamics. One of the mtDNA maintenance proteins is MPV17, which is a hydrophobic membrane protein involved in nucleotide metabolism but ist molecular function is unknown. To date, 48 MPV17 pathogenic variants are known. The vast majority of affected individuals present with an early-onset encephalohehepatopathic disease characterized by hepatic and neurological manifestations, with no therpeutical option apart from liver transplantation into affected infants. The molecular mechanisms causing the disease are largely unknown. It has been published that the Mpv17 protein is a mitochondrial membrane protein and hence mutations in it impair the transport of nucleosides into the mitochondria. However, we earlier presented data showing that the Mpv17 protein is not localised to the mitochondrial membrane. Here we corroborate in human cells HAP 1 cells and demonstrate specificity by showing that human Mpv17 knockout cells loose an immunoflorescence and western blot signal with our anti- Mpv17 antibody. Therefore, we hypothesise, that in Mpv17 mutation caused human genetic disease mitochondrial nucleoside import is not impaired, and therefore nucleoside bypass thechnology, which has been proven successful in TK2 deficient mice and humans should be beneficial in Mpv17 deficiencies too.

Bioorthogonal Fluorogenic Probes – Application in Live Cell Imaging and Single Molecule Localization Microscopy

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Small-molecule fluorophores with tailor-made photophysical properties have gained prime importance for modern microscopy technologies. Ideally a fluorescent probe should have excellent target specificity (low background), high photostability and photon output as well as suitable spectral properties for the microscopy technique of choice and the biological question to study. Fluorogenic probes for bioorthogonal labeling chemistry are highly beneficial to improve target specificity and to reduce background signal in fluorescence microscopy imaging.[1]

We recently reported a palette of fluorogenic dyes for intracellular live cell protein labeling.[2] Now, we present novel, further improved fluorogenic dyes and demonstrate their utility in multi-color live cell imaging under no-wash conditions. Moreover, we merged our concept of fluorogenicity with fluorophores that exhibit spontaneous blinking based on intramolecular spirocyclization at physiological pH.[3] We successfully applied these fluorogenic and spontaneously blinking fluorophores in live cell single-molecule localization microscopy (SMLM) with a localization precision of ~20 nm. Finally, we visualized biomolecular interactions based on proximity-driven bioorthogonal chemistry utilizing a bifunctional fluorogenic probe.[4]

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Live-cell protein labeling with nanometer precision by cell squeezing

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Many dynamic biological processes are regulated by protein–protein interactions and protein localization. With this regard, chemoselective and site-specific labeling of proteins evolved to a central aspect in chemical biology and cell biology. Protein labeling demands for high specificity, rapid as well as efficient conjugation with ultra-bright spectroscopic probes. Generic methods that do not interfere with the proteins´ function, dynamics, subcellular localization, or crosstalk with other factors are crucial to probe and image proteins *in vitro* and in living cells.

Here, we report on recent applications of small lock-and-key pairs, namely the multivalent chelator head *tris*NTA and nanobodies, for the labeling of proteins in living cells with nanometer precision.[1] By a vector-free microfluidic device as control system, we enabled their high-throughput, fine-tuned delivery of nanomolar concentrations. The scalable and high-throughput strategy allowed targeting and mapping multiple proteins simultaneously in living cells with the resolution of micro- to nanometers.The fast and fine-tuned intracellular transfer by microfluidic cell squeezing resulted in low background, low toxicity, and high-throughput. The high-affinity interaction pairs enabled fast labeling (≤10 min) at subnanomolar concentrations with tunable labeling density, whichsubstantially improved the localization accuracy in live-cell super-resolution microscopy. The *in-situ* photo-activation even allowed the labeling at defined time points and dynamic cellular imaging. Collectively, our small lock-and-key elements will help to investigate and visualize protein localization and dynamics in living systems with subnanomolar concentrations.

Intravital STED microscopy of synaptic proteins in the mouse cortex

Presenting author: **Katrin Willig**

Far-field light microscopy is a powerful technique for imaging structures inside living cells, tissue or living animals. However, fine details or substructures of the cell cannot be visualized with conventional light microscopy because of the diffraction-limited resolution (~ 200 – 350 nm). This barrier had been overcome by a whole family of super-resolution microscopy or nanoscopy concepts such as STED, RESOLFT, PALM, STORM etc. Amongst these techniques, STED microscopy stands out for its fast recording speed; its inherent 3D sectioning capability; the potential to image deep within tissue; and the possibility of exploiting a vast array of commercially available fluorescent dyes including standard fluorescent proteins, such as GFP, YFP or red-emitting fluorescent proteins. Moreover, the recorded image is a linear response of the number of fluorophores without any mathematical processing being involved. Therefore, STED microscopy is ideally suited for imaging within tissue and as such for imaging and quantifying morphological changes in the living mouse brain.
I will present applications of STED microscopy to image neuronal structures in the brain of living mice. We image the cerebral cortex of a living mouse through a glass window, so that we can observe the dynamics of dendritic spines in the molecular layer of the visual cortex. We had superresolved actin and its morphological changes in the cortex of an anaesthetized mouse, which was the first STED microscopy of a dendritic sub-structure in a living mouse. Recently, we have pioneered in vivo superresolution of the postsynaptic scaffolding molecule PSD95, one of the key components in the organization of synapses that is thought to control synaptic strength by anchoring postsynaptic receptors. We have shown for the first time the dynamic organization of PSD95 over several hours in the visual cortex of a living mouse.
These results show that STED nanoscopy is a highly suitable tool for research in neuroscience which can play a substantial role in the study of learning and memory.

Multi-scale modeling of cryo-EM protein structures using graph theory

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We developed a graph-based description of large protein structures, mainly explored by cryo-EM. Based on a previous modeling of protein secondary structure topology (Protein Topology Graph Library - PTGL, [1, 2]), we defined complex graphs that describe the chain topology of large protein complexes. PTGL is an online database and holds *protein graphs* and *complex graphs* of 123,505 PDB structures.
The number of newly resolved protein structures not only increases rapidly but also the structures become bigger and more complex. That makes the application of automated methods at different scales necessary. The representation of protein structures as graphs enables visual and computational analysis. In the case of protein complexes represented as complex graphs, a high vertex degree and betweeness centrality identify protein chains that play a vital role in the architecture of the protein complex. The abstraction reflects structural symmetries in the graphs. *Large structures* are of special interest as they usually comprise whole structures in their biologically active state, for example, viral capsids or large enzymes. They are typically available in the *mmCIF* format. We implemented an efficient parser of the *mmCIF* format to derive and analyze the corresponding graphs, enabling the survey of such large protein complexes for classification and motif search.

[1] Schäfer *et al*., Bioinformatics, 2016
[2] Koch & Schäfer, Curr Opin Struct Biol, 2018

Novel imaging techniques to study the role of cGMP in cancer progression

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Epidemiologic studies showed that the use of the cyclic guanosine monophosphate (cGMP)-elevating drug sildenafil (Viagra®) correlates with melanoma incidence. Indeed, we and others have identified a cGMP-dependent growth-promoting signaling pathway in melanoma cells. Since under *in vivo* conditions tumorcells are constantly interacting with their neighboring stroma cells, we developed imaging techniques to analyze the complex interplay of cGMP signaling in tumor and stroma cells under close-to-native conditions. (1) Mice that express the FRET-based cGMP biosensor cGi500 allow for monitoring of cGMP signals in individual cells in their native environment. (2) Positron emission tomography (PET), on the other hand, is a powerful tool to follow cell growth and distribution throughout the body in a macroscopic and noninvasive manner. As proof-of-principle, we have successfully used our PET reporter mice to track platelets, $CD4^+$ T cells and cardiomyocytes in longitudinal studies *in vivo*. In addition, we have visualized biochemical cGMP signals by FRET microscopy in tumor cells *in vitro* and, importantly, in living tumor tissue. Combination of (1) microscopic visualization of cellular signaling in living tumor samples (cGMP imaging) and (2) noninvasive monitoring of cellular distribution within the body (PET imaging) will increase our understanding of tumor progression in the presence of complex tumor-stroma-interactions.

Watching cGMP signalling live and in colour

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Author(s):
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Biochemical reactions are carefully regulated in space and time. Accordingly, it is very informative to monitor the spatiotemporal dynamics of cellular signals and their functional outcome in real-time in close-to-native environments. Our work focusses on the second messenger 3',5'-cyclic guanosine monophosphate (cGMP) that plays an important role in many physiological functions from vasodilation to learning and memory to phototransduction to regulation of cell growth and survival. Dysfunctions of cGMP signalling are also associated with various diseases, such as heart failure, atherosclerosis, retinal degeneration, or tumour formation. We have developed transgenic mice that express a FRET-based biosensor, called cGi500, either in the whole animal or only in specific cell types. This enables us to visualise cGMP concentration changes evoked by endogenous hormones or cGMP-elevating drugs (e.g. Viagra®) in primary cells, isolated tissues and even anaesthetised mice in real time with high spatiotemporal resolution. Using correlative imaging of cGMP signals and cell phenotype at the single-cell level, we found that cell populations are highly heterogeneous with respect to cGMP responses and that the "cGMP status" of a given cell correlates with its differentiation state. In sum, we show that the combination of mouse genetics and FRET imaging provides new opportunities to investigate the biochemistry of living mammalian cells, tissues, and animals.

Digital Pathology of Hodgkin lymphoma: analysis of CD30-stained whole slide images using graph theory

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In the course of diagnosis, pathologists manually inspect morphological properties of specific cells and their spatial distribution. The high number of data makes the analysis of whole slide images (WSI) challenging.

We developed an approach for the hybrid analysis of immunohistologically CD30-stained tissue sections, bringing together the fields of computer vision and network theory [1]. Based on WSI of 35 patients either diagnosed with Hodgkin lymphoma or lymphadenitis, we explored diagnostic relevant features, e.g. the number of CD30-positive cells and their spatial distribution.

We implemented an image analysis pipeline for the exploration of WSI [2], which performs a pixel-based examination of the layer with a low resolution and an object-based analysis of the layer with a high resolution. For the object-based analysis, we developed a pipeline for the detection of CD30-positive cells and their morphological features, e.g. area and circumference [2]. The subsequent abstraction of WSI as a labeled network of interacting cells with morphological features allows an

investigation of the cell distribution with simultaneous reduction of data. Thus, for instance, we have been able to reveal first differences between the cell graphs of lymphadenitis and Hodgkin lymphoma by evaluating established graph properties, e. g. node degree distribution.

[1] Yener, Comm. ACM, 2016
[2] Schäfer et al., Bioinformatics, 2015

Insight into mechanism, assembly and disease from cryo-EM structures of respiratory complex I

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NADH:ubiquinone oxidoreductase (complex I) plays a key role in aerobic energy metabolism and is one of the largest membrane protein complexes known (*1*). Redox-linked proton translocation by complex I generates 40 % of the proton-motive force driving ATP synthase. The critical steps of energy conversion are associated with ubiquinone redox chemistry. We have determined cryo-EM structures of complex I from the aerobic yeast *Yarrowia lipolytica* in the deactive state and under turnover conditions (*2*). The ubiquinone binding site provided clues on the mechanism of redox-linked proton translocation. At an improved resolution of 3.2 Å, we observe a ubiquinone molecule entering its access path from the membrane into the hydrophilic peripheral arm. Complex I dysfunction causes a large number of neuromuscular and neurodegenerative diseases (*3*). The accessory subunit NDUFS4 was identified as a hot spot for pathogenic mutations causing Leigh syndrome, a serious neurological disorder (*4*). To understand the structural basis of NDUFS4-linked Leigh syndrome we have solved the cryo-EM structure of mutant complex I from *Y. lipolytica* lacking the orthologous subunit. Complex I assembly is an intricate multi-step process aided by numerous assembly factors. Assembly factor NDUFAF2 is a paralog of accessory complex I subunit NDUFA12. We have solved the cryo-EM structure of an assembly intermediate that includes the NDUFAF2 assembly factor (*5*).

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