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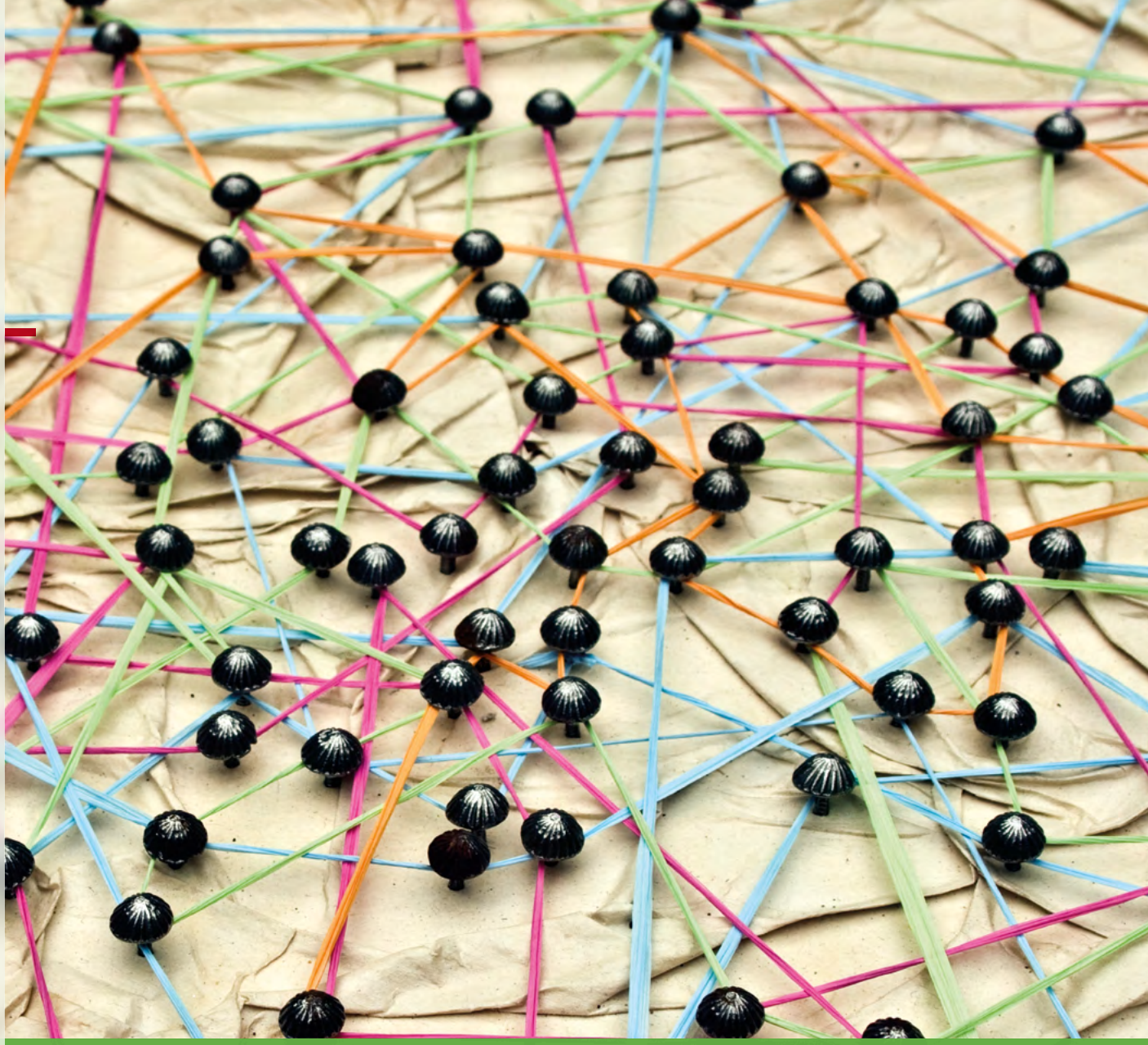


BIOSS

PROJECTS & METHODS

bioss
CENTRE FOR BIOLOGICAL SIGNALLING STUDIES

Preface

Photograph by: Goethe-Uni/Dettmar

The BIOSS Centre for Biological Signalling Studies conducts signalling research from analysis to synthesis. In the last eight years, it has established itself as an important partner for the biological-medical research community in Freiburg, in Germany, and in the world. BIOSS was one of the first to combine the large field of biological signalling research with the emerging field of synthetic biology. This approach is now an important teaching and research subject of the University of Freiburg and has promoted a multitude of collaborations within and outside Freiburg.

BIOSS not only recruits new scientists to Freiburg, but has funded 77 scientific projects through its research budget. The scientific success of our programme is documented by more than 200 BIOSS publications per year, including many joint publications in outstanding journals. That BIOSS is an active research community was easy to see at our last annual BIOSS retreat in June 2015 in the town of Schluchsee. We had lively discussions and numerous interactions between the participants.

To further foster the collaborative spirit of our Cluster of Excellence, the BIOSS office has assembled a collection of BIOSS projects and methods in this BIOSS-PM booklet. My hope is that this book will further foster the collaboration within BIOSS and increase the visibility of our science and diverse methodologies. Given the ongoing success of BIOSS, I am confident that BIOSS will also play an important part in the next round of the excellence initiative.

I want to thank the whole BIOSS office for doing such a superb job in administrating our centre and our press officer, Katrin Albaum, for assembling this book, which we hope will be useful for the BIOSS community in the future.

Prof. Dr. Michael Reth

www.bioss.uni-freiburg.de

Content

Pharmacology and Toxinology

HEAD Prof. Dr. Dr. Klaus Aktories

To prevail in a hostile environment, many pathogenic bacteria produce a huge armamentarium of toxins to manipulate and hijack signalling of host cells. In most cases, the bacterial toxins are enzymes, which are characterized by extremely high potency and selectivity. Preferred substrates of toxins are GTP-binding proteins, which act as molecular switches in cell signalling. Another frequent target of bacterial toxins is the cytoskeleton of host cells. The toxins manipulate target proteins by various types of posttranslational modifications, including ADP-ribosylation, glycosylation, AMPylation, deamidation, or proteolytic cleavage[1].

Major aims of the research group are to clarify the structure-function relationships of the toxins, to analyse their receptor-binding, up-take, molecular mechanisms, and to elucidate the functional consequences of toxin actions. Further objective is the development of bacterial protein toxins for usage as pharmacological tools in synthetic biology.

PROJECTS

Toxins acting on Rho-GTPases

We study various bacterial protein toxins, which target Rho GTPases, a family of Ras-like proteins, which are involved in numerous signalling processes. Rho GTPases are switch proteins and master regulators of the cytoskeleton. They are involved in cell motility and intracellular traffic, phagocytosis and secretion, in cell cycle progression and tumour metastasis. Bacterial toxins activate or inactivate Rho GTPases. Specific research focus is on *Clostridium difficile* toxins A and B, which are the causative agents of antibiotic-associated diarrhea and pseudomembranous colitis. These nosocomial diseases are emerging medical threats in clinical settings. Toxins A and B inactivate Rho proteins by glucosylation of threonine35/37[1]. Rho proteins are also inhibited by the insecticidal *Photorhabdus* toxin PaTox, which causes GlcNAcylation of a conserved tyrosine residue of Rho[2]. Rho proteins are activated by cytotoxic necrotizing factors (CNFs) from *E. coli*. CNFs are major virulence factors of uropathogenic *E. coli* and play a role in meningitis caused by *E. coli.* CNFs deamidate a conserved glutamine residue (Gln61/63) of Rho proteins, thereby persistently activating the GTPases[3]. Also the insecticidal *Photorhabdus* toxin TccC5 activates Rho proteins; however, this toxin modifies Gln61/63 by ADP-ribosylation[4].

bioss.science/pm/pharmacology_toxinology

Toxins acting on heterotrimeric G proteins and elongation factor 1A

Another topic of research is *Pasteurella multocida* toxin (PMT), which activates heterotrimeric G proteins of the Gi, Gq and $G_{12/13}$ family by deamidation [5]. This toxin inactivates osteoblasts and stimulates osteoclasts, thereby, causing destruction of bones. A similar activation of heterotrimeric G proteins is achieved by the deamidase domain of *Photorhabdus* toxin PaTox. Elongation factor EF1A, which is essential for protein synthesis, is targeted by glucosyltransferases (Lgts) of *Legionella pneumophila*, the cause of Legionnaires disease. This intracellular pathogen inhibits protein synthesis of host cells by glucosylation of serine-53 of EF1A. Goal of current studies is the clarification of the precise molecular mechanism of protein synthesis inhibition.

Table 1. Bacterial toxins as cell-biological tools

Toxin	Source	Target (consequence)	Activity	Cellular uptake	Receptor	Concentration
CNF1	E. coli	Rho GTPases (activation)	Deamidase	+	BCAM, p67LBP	1 nM
CNFY	Yersinia pseudotuberculosis	Rho GTPases (activation, preferentially RhoA,B,C)	Deamidase	+		1 nM
YopT	Yersinia ssp.	Rho GTPases (inactivation)	Protease	-		
Toxin B	Clostridium difficile	Rho GTPases (inactivation, Rho, Rac, Cdc42)	Glucosyltransferase	+		0.1 – 100 pM
Toxin A	Clostridium difficile	Rho GTPases (inactivation, Rho, Rac, Cdc42)	Glucosyltransferase	+		100 pM – 1 nM
TpeL	Clostridium perfringens	Ras (inactivation, Rac at high dose)	Glucosyltransferase	+	LRP1	0.1 – 10 nM
PaTox^G	Photorhabdus asymbiotica	Rho GTPases (inactivation)	GlcNAc-transferase	+*		100 nM
PaTox^D	Photorhabdus asymbiotica	Activation of Gαi, Gαq/11	Deamidation	+*		100 nM
Lgt1-3	Legionella pneumophila	Elongation factor 1A (inhibition of protein synthesis)	Glucosyltransferase	-		
PMT	Pasteurella multocida	Heterotrimeric G proteins Gαi, Gαq/11, Gα12/13 (activation)	Deamidation of a conserved glutamine residue	+		0.1-10 nM
PMT variant	Chimeric toxin	Heterotrimeric G proteins altered specificity (G-protein activation)	Deamidation of a conserved glutamine residue	+		1-30 nM
TccC3	Photorhabdus luminescens	Actin (polymerization)	ADP -ribosyltransferase	+*		2-20 nM
TccC5	Photorhabdus luminescens	Rho GTPases (activation)	ADP -ribosyltransferase	+*		2-20 nM
CDT	Clostridium difficile	Actin (depolymerization)	ADP -ribosyltransferase	+	LSR	0.1-10 nM
C3	Clostridium botulinum/ limosum	RhoA, B C (inactivation)	ADP -ribosyltransferase	-		1-10 nM
C2IN-C3	Chimeric toxin: Clostridium botulinum/ limosum	RhoA, B, C (inactivation)	ADP -ribosyltransferase	+		1-10 nM

*With protective antigen (PA) from Bacillus anthracis (receptors: TEM8 and CMG2)

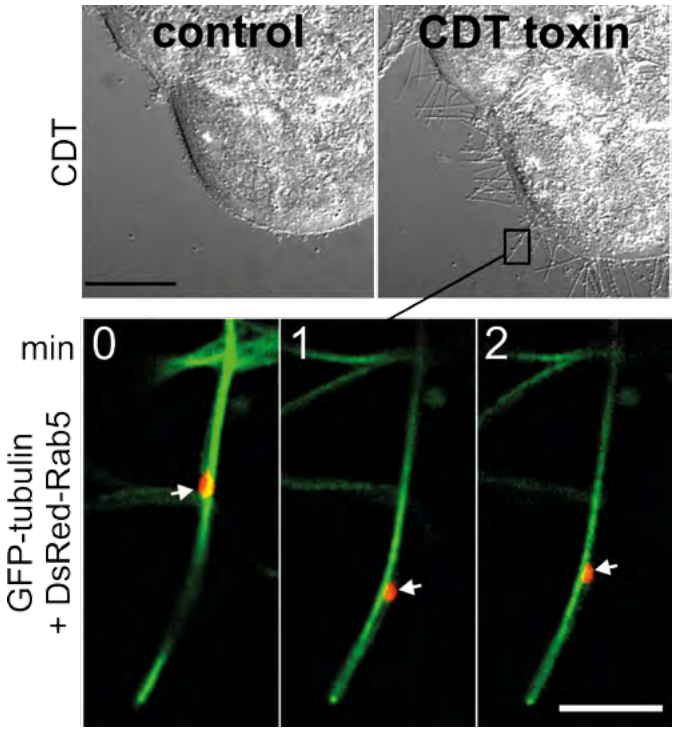
CNF1 (Cytotoxic necrotizing factor1) activates several Rho GTPases by deamidation. The toxin is taken up via two receptors BCAM (basal cell adhesion protein) and p67LBP (p67 laminin binding protein). CNFy is more specific for RhoA, B and C than CNF1. The protease YOPT (*Yersinia* outer membrane protein T) inactivates Rho proteins by cleavage at a C-terminal cysteine residue that is isoprenylated and responsible for membrane binding. *C. difficile* toxins A and B inactivate Rho proteins by glucosylation at a threonine (Thr35/37) residue. TpeL is related to *C. difficile* toxins A and B but Ras is the preferred target, which is inactivated by GlcNAcylation at Thr35. The toxin is taken up via low density lipoprotein receptor related protein 1 (LRP1). *Photorhabdus asymbiotica* toxin PaTox possesses two toxic domains: The PaTox^G domain harbors a glycosyltransferase, which modifies Rho GTPases at tyrosine-32/34. The PaTox^D domain possesses deamidase activity and activates heterotrimeric G-proteins. Lgt1-3 are related *Legionella glucosyltransferases* that inactivate elongation factor EF1A by glucosylation at serine-53. PMT (*Pasteurella multocida* toxin) activates various heterotrimeric G proteins by deamidation. PMT-related chimeric toxin possesses high substrate specificity for $G\alpha_i$. The ADP-ribosyltransferase domains of *Photorhabdus* toxins TccC3 and TccC5 are transported into target cells by the protective antigen (PA), which is the binding and delivery unit of anthrax toxin. CDT (*Clostridium difficile* transferase) is a binary actin-ADP-ribosylating toxin, which is taken up into target cells via lipolysis-stimulated lipoprotein receptor (LSR). The ADP-ribosyltransferase *Clostridium botulinum* C3 toxin is efficiently taken up only by few cell types (e.g., macrophages). Therefore, a chimeric toxin (C2IN-C3) is generated, which uses the binding and up-take unit of *C. botulinum* C2-toxin.

Toxin acting on the actin cytoskeleton

Other bacterial protein toxins target the actin cytoskeleton. Binary toxins from Clostridia and Bacillus species, which consist of separate binding and enzyme components, depolymerize actin by ADP-ribosylation at arginine177. Major toxin of this group is *C. difficile* transferase CDT, which is produced by hypervirulent *C. difficile* strains. These actin-ADP-ribosylating toxins have not only effects on the actin cytoskeleton but also disturb the microtubule system of target cells[6]. In contrast to the actin depolymerizing effect of binary toxins, *Photorhabdus luminescens* toxin TccC3 ADP-ribosylates actin at threonine148, which results in actin polymerization[4].

METHODS

The toxins mentioned above are not only important virulence factors and pathogenic agents but also highly potent and specific pharmacological tools to manipulate cell signalling of target cells under experimental conditions. Using the toxins, specific cell signal pathways can be switched on and off. By construction of toxin chimeras, specific cell targeting is possible. The toxins possess elaborate cell up-take mechanisms and can be used as transport system to deliver proteins of different functions into target cells.



Upper panel: Caco-2 cells were intoxicated by *C. difficile* toxin CDT, which ADP-ribosylates actin. After 2 hours, microtubule-based cell protrusions developed. Lower panel: The toxin-induced cell protrusions exhibit vesicle transport as shown by transfection of fluorescence-labelled tubulin (GFP-tubulin) and Rab protein (dsRed-Rab5). Bar 5 µm.
Source: Dr. Carsten Schwan / Research group Aktories

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Membrane Biophysics

HEAD Prof. Dr. Susana Andrade

PROJECTS

Molecular mechanism of NH_4^+ transport across biological membranes

Our group is interested in dissecting the molecular and atomic details of protein-mediated ammonium translocation across biological membranes. These Amt (Ammonium transport) proteins form trimeric arrays in the membrane, with a conserved core of 33 transmembrane helices, to selectively recruit and transport the cation NH_4^+. Despite the availability of high-resolution crystal structures from bacterial Amts to the human RhCG (Rhesus proteins are the eukaryotic Amt homologs), the mechanistic details of transport is not known, essentially due to the practical difficulties to distinguish and detect NH_4^+/NH_3. We have established an *in vitro* electrophysiological assay using pure protein that allows us to analyse the electrogenic characteristics of protein-mediated transport. Specifically, we confirmed that Amt proteins are electrogenic NH_4^+ transporters and not NH_3 gas channels and characterized the functionality of a number of wild-type proteins. Currently, we investigate the behaviour of selected point mutants to prove the role of particular amino acid residues in various stages of the transport process.

Regulation of NH_4^+ transport by protein: protein interaction

GlnK proteins belong to a broad family of PII proteins that sense the cellular levels of 2-oxoglutarate, glutamine and the ATP:ADP ratio to regulate gene expression and enzymatic activities related to N-assimilation events. In particular, in the presence of ATP, GlnKs can form an inhibitory complex with Amt proteins while ATP:2-oxoglutarate is an effector for complex dissociation instead. Our studies detailed the thermodynamic aspects of effectors binding to GlnK, highlighted the consequent conformational switches occurring in the protein and allowed us to explain the molecular reasons for complex formation and dissociation. Our present focus is to understand how subtle amino acid differences among GlnK homologs account for the observed variations in effector sensing and GlnK response. Obtaining structures of Amt:GlnK complexes is also in our horizon.

Ammonium signalling

Amt proteins can also occur associated with outer-membrane signal transducing domains. To confirm their NH_4^+ transport and/or sensing function, we use electrophysiology tools. To characterize the transducing domains, we combine molecular biology with *in vitro* radioactive techniques, mass spectrometry, high-performance liquid chromatography, or stopped-flow assays. To access the signal transduction events involving conformational changes, we use X-ray crystallography, small angle X-ray scattering and spin labelling tools.

bioss.science/pm/membrane_biophysics

METHODS

Electrophysiology

This method allows following the flow of ions/charges – in the form of currents – across two compartments separated by a lipid bilayer. Typically, naked bilayers are impermeable to ions but the presence of specific membrane proteins introduces selective permeability. *Planar lipid bilayer electrophysiology* is a powerful technique that monitors single (single channel currents) or multiple protein units (macroscopic currents) fused to a small lipid bilayer area that is painted on a 100-400 µm diameter bore. Current changes can then be measured in the presence of the selected ionic specie(s) with or without the application of voltage differences between the two compartments. The technique is very useful to characterize channels or transporters with high ionic conductance (i.e., high ion flow rates) (Lü *et al.* 2012a; Lü *et al.* 2012b, Lü *et al.* 2011). For slow conducting transporters, *solid-supported based-electrophysiology* is instead the technique of choice: This technique relies on the adsorption of a suspension of liposomes or proteoliposomes (eventually carrying a high number of protein units in each vesicle) to a hybrid bilayer that sits on top of a gold electrode. The magnification of each protein contribution allows recording transient currents after a rapid exchange of the surrounding solution (Wacker *et al.* 2014).

The techniques described above can be used with membrane protein targets that were *purified* in the presence of detergents and, at a later stage, *reconstituted* back into a lipid environment, often large unilamellar vesicles (LUV) or liposomes (Wacker *et al.* 2014).

Crystallography

A pure protein solution can be induced to form crystals if the solubility of the protein decreases and crystal nucleation occurs. When well-ordered crystals are obtained, they can be cryo-protected, collected, and subject to a well-defined X-ray beam (at a in-house diffractometer or synchrotron source) so that a number of diffraction images can be collected at various crystal rotation angles. This data set is analysed in the computer to yield a final three-dimensional electron density map, into which a protein model can be built (Andrade *et al.* 2005). The technique can provide models at the highest detail levels, with resolutions extending below one Ångstrom.

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The Planar Lipid Bilayer Workstation. Photograph by: Katrin Albaum

Bioinformatics and Molecular Genetics

HEAD Prof. Dr. Ralf Baumeister

PROJECTS

Insulin signalling in tumorigenesis

We study stem cell tumorigenesis (hyperplasia, cell migration, and tissue invasion) using the nematode *C. elegans* and human cell cultures as models. In detail, we have established worm models of germline stem cell tumours encompassing tumour-suppressive, but also oncogenic properties of the FOXO transcription factor DAF-16, a major downstream effector of insulin/IGF (IIs) signalling in worms and humans. During the past funding period of BIOSS, we have described novel oncogenic, non-cell-autonomous activities of FOXO and have identified novel inhibitors and enhancers of tumorigenesis, including genes that function in pathways that crosstalk with IIs. We have also found that dysfunction of the tumour suppressor gene PTEN/DAF-18 may result in stem cell tumours, depending on an earlier nutritional status of the organism.

Mitochondrial stress responses

Using genome-wide screenings, we have identified 55 candidate genes that contribute to the mitochondrial stress response upon application of reactive oxygen species that impair mitochondrial electron transport chain activities. We have proposed mechanisms how the mitochondria respond to oxidative stress through activating mitochondrial chaperone synthesis, and have identified a kinase that links endoplasmic and mitochondrial stress responses. Our work on mitochondrial stress also translates to a better understanding of genes involved in neurodegenerative disorders, most notably Parkinson's Disease.

Signalling function of Parkinson's disease genes

We have shown functional relationships of several genes, whose mutations segregate with hereditary Parkinson's Disease. Most notably, we showed antagonistic activities of PINK1 and LRRK2 and their *C. elegans* orthologues, and have identified signalling mechanisms to understand their functional relationships. Our findings that intracellular membrane transport is diversely affected by PD genes have allowed us to screen for modulators of PD gene dysfunction, the results of which we are currently investigating.

Crosstalk between insulin and TOR signalling in tumorigenesis and stress response

Using both animal models (*C. elegans*) and cell culture, we identified several new functional connectivities of insulin/IGF and TOR signalling with relevance to human cancer. In particular, we identified novel regulators and interactors of mTORC using large-scale mass spectrometry approaches, and a previously unknown crosstalk between mTORC and TGFß signalling.

bioss.science/pm/bioinformatics_molecular_genetics

METHODS

C. elegans disease/signalling models

The work-horse of our studies is the model organism Caenorhabditis elegans, which allows us to set up assays for the functional characterization of novel components of signalling pathways, evaluation of gene function, the generation of synthetic models encompassing a living animal system supplemented with human genes of interest that integrate seemingless.

CRISPR/Cas9 genome editing

We have developed several protocols that allow us successfully to generate genomic deletions (gene knock-outs), but also single base-pair alterations of genomic regions (genome editing, e.g. to change single amino acids in a protein) in *C. elegans.* This method has been used to systematically introduce loss-of-function and gain-of-function (e.g. constitutively active proteins, or to generate mutations at positions implicated with hereditary diseases) mutations in the genome of *C. elegans.* Thus, we generated for example point mutations resulting in amino acid exchanges of proteins homologous to mutants involved in human disease. We are currently expanding these protocols to allow integration of reporter genes at dedicated sites of the genome, to generate *in vivo* tagged proteins, or to genomically integrate entire human genes under the control of a *C. elegans* promoter.

Synthetic organisms for pharmacological drug screenings

We can generate synthetic organisms that allow drug screenings against human proteins/mutants in the functional environment of an entire organism. We have used a variety of methods, e.g. to integrate human Parkinson's Disease variants into the worm, and have used this synthetic organism to screen for drugs ameliorating mutant phenotypes. Our lab has experience in screenings for up to 6,000 compounds in medium-scale settings, but also ample experience in upscaling such screens to higher throughput.

FACS-like sorting of entire organisms

We run a FACS-like *C. elegans* sorter that allows us high-throughput sorting of living specimens according to several criteria: length, density, structural abnormalities, expression of marker proteins, fluorescence, lipid content, etc. Dual sortings and resortings, as well as subsequent distribution of candidates to microtiter plates are also possible.

Proximity proteomics and labelling

Methods for the *in vivo* labelling of protein nano-environments. We are employing proximity proteomics approaches that were first introduced into the BIOSS portfolio by Prof. Dr. Michael Reth's group, with which we have established several collaborations. For tandem-tag of proteins, our lab has developed the Snavi-Tag system that we have made available to the public through ADDGENE.

The roundworm *C. elegans* is only 1 mm long and one of the most favoured laboratory models for the genetic study of signalling paths.
Images: Ralf Baumeister, Photomontage: Katrin Albaum

Genome-wide screenings for modulators of gene function

Our main model organism *C. elegans* is well-known for genome-wide screenings, e.g. to identify novel pathway components, modifiers of mutant dysfunction, alterations of behaviour and more. We own several libraries for RNA interference of basically every *C. elegans* gene and have used these libraries successfully to identify novel gene functions and interactions. Moreover, we generated sub-libraries of e.g. all kinases and all transcription factors encoded in the genome. Using our bioinformatics platform, rapid generation of novel sub-libraries, e.g. phosphatase libraries, or GPCR genes, are easily doable.

Behavioural analyses

C. elegans is a model very well-known to couple neurobiological functions to even complex behavioural outputs, including motility, mating behaviour, learning paradigms, sensation of olfactory, chemical, mechanical, osmotic environmental signals. We have a tool-box of more than 150 phenotypic readouts that we can employ to even address complex functions of the nervous system or whole organisms.

Stress biology and endomembrane transport dynamics

We have invested in the last years in many tools to monitor mitochondrial dynamics and functions, and to investigate endomembrane transport properties, lysosomes and ER functions, protein aggregation and ROS stress, intracellular sorting dynamics, necrosis and apoptosis, phagocytosis and autophagy. Our toolbox includes models in both *C. elegans* tissues and human cell cultures.

Interactor screenings

Our group has implemented tools to study protein interactions using the split-ubiquitin system that allows identification of binary or tertiary interactions in the living cell. This method has been stream-lined to particularly allow monitoring interactions of transcription factors and of membrane-bound proteins, interactions of the latter are notoriously difficult to analyse. We have established or acquired proteome libraries for screenings of human brain, human fetal brain, kidney, different stages and conditions of *C. elegans*, as well as of other model organisms, including plants. Automation of these procedures have been initiated in collaboration with ZBSA and the University of Marburg. Moreover, we have established protein interaction screenings using mass spectrometry tools. These have been used extensively in the past year to identify novel interactors of human stress signalling and metabolic pathways, and of *C. elegans* proteins.

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Biochemistry and Molecular Biology • Mitochondrial Biogenesis

PROJECT LEADERS

1. PD Dr. Thomas Becker
2. PD Dr. Martin van der Laan
3. Prof. Dr. Nikolaus Pfanner
4. Prof. Dr. Nils Wiedemann

PROJECTS

Mitochondria are of central importance for cellular energetics, metabolism, and signalling. Mitochondria have been derived from an ancestral prokaryote related to alpha-proteobacteria engulfed by a primordial eukaryotic cell. Though initially quasi-autonomous endosymbionts, mitochondria became more and more integrated into the genetic and metabolic system of the eukaryotic host cell. Recent studies reveal that mitochondria are deeply integrated into a majority of cellular processes. Our groups study the biogenesis, function, and dynamics of mitochondria in projects funded by BIOSS, Collaborative Research Centres, Graduate Schools, and individual grants. Mitochondria contain more than 1,000 different proteins. About one percent of the proteins (13 in humans and 8 in baker's yeast) are encoded by the genome in the mitochondrial matrix and are synthesized on ribosomes inside mitochondria. The remaining, large majority of mitochondrial proteins are encoded by nuclear genes and are synthesized on cytosolic ribosomes. The precursor proteins carry targeting signals that direct them to specific receptors on the mitochondrial surface, termed translocase of the outer mitochondrial membrane (TOM) receptors. Upon translocation through the protein import channel formed by Tom40, the precursor proteins are sorted to distinct intramitochondrial locations. Depending on the type of the targeting signals (N-terminal positively charged signals or different kinds of internal targeting signals), different translocase machineries direct the precursor proteins to the mitochondrial subcompartments outer membrane, intermembrane space, inner membrane, or matrix. Major questions are how the molecular machineries that transport precursor proteins cooperate with each other and with the bioenergetics apparatus of mitochondria, how the recognition and transfer of precursor proteins are coordinated, how protein transport is connected to the dynamics of the mitochondrial membranes, and how mitochondria communicate with other cellular compartments.

METHODS

Yeast plasmid shuffling

To generate temperature-sensitive mutant strains of essential genes or respiratory competent deletion strains, plasmid shuffling is employed in baker's yeast (*Saccharomyces cerevisiae*). The chromosomal gene of interest is covered by a plasmid borne copy before the chromosomal copy of the gene of interest is disrupted. Next, a second plasmid containing truncated or mutated copies (e.g. generated by error prone PCR) of the gene of interest is transformed into the deletion strain. Subsequently cells are screened for loss of the wild type gene-containing (covering) plasmid by incubation with a substrate, which is converted into a suicide inhibitor by the product of the marker gene. In case no second plasmid is transformed, this procedure will generate a deletion strain by plasmid shuffling. The growth behaviour of the strains is subsequently analysed on solid media at different growth temperatures (Kutik *et al.* 2008).

Subcellular fractionation

To separate different cellular fractions, subcellular fractionation by differential centrifugation is employed. As basic protocol, the postnuclear supernatant (PNS) is fractionated into a crude mitochondrial P 13,000 x g fraction, a microsomal P 100,000 x g fraction and a cytosolic S 100,000 x g fraction. The enrichment of cellular fractions is controlled by Western blotting with antibodies against organelle-specific marker proteins (Wiedemann *et al.* 2006).

Isolation of purified mitochondria

Mitochondria are isolated by differential centrifugation und are subsequently purified by gradient centrifugation (Meisinger *et al.* 2006).

Respiratory activity

To analyse the basal and maximal respiration of mitochondria or cells, the Oxygraph (Oroboros) or the Seahorse extracellular flux analyser (Seahorse bioscience) are employed. In both systems, the oxygen concentration is measured in the presence of respiratory substrates or inhibitors to determine the respiratory rate of wild type and mutant cells or mitochondria (Böttinger *et al.* 2013; Mossmann *et al.* 2014).

Mitochondrial sublocalization

The sublocalization of mitochondrial proteins is determined by protease accessibility assays of intact mitochondria, outer membrane disrupted, and solubilized mitochondria. Membrane association is probed by sonication and separation of the membrane fraction under increasing salt concentrations. Membrane integration of proteins is assessed by carbonate extraction using different alkaline pH values (Wiedemann *et al.* 2006).

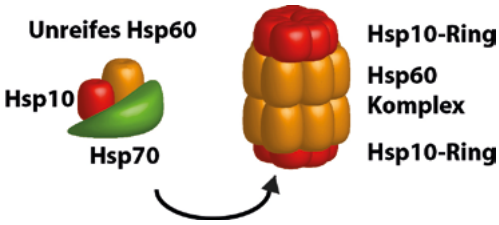
Affinity purification

Protein-protein interactions are crucial for the function and regulation of protein machines. Systems to detect protein-protein interactions within a cellular or mitochondrial lysate are therefore crucial to obtain insight into the organization of complex protein machineries. We have established the chromosomal fusion of genes of interest to small affinity tags like His-, HA- and Protein A-tag in *S. cerevisiae*. The affinity purifications using the corresponding affinity matrices are optimized in terms of detergent/protein ratio and incubation time. Co-purification of proteins indicates interactions or the presence in the same protein complex. To obtain optimal purification results and a comprehensive overview about partner proteins, we have affinity-tagged several components of a single protein complex. The combination of cross-linking and purification under denaturing conditions allows the identification of close interactions between two proteins. We have established purification and elution under non-denaturing conditions to study the interaction of protein complexes by blue native electrophoresis (Böttinger *et al.* 2013; Qiu *et al.* 2013).

Blue native electrophoresis

We use blue native electrophoresis to study protein complexes in isolated mitochondria. Non-ionic mild detergents like digitonin are suited well to analyse protein complexes. We have optimized the solubilisation conditions for mitochondrial membranes and remove unsoluble material by centrifugation. We have successfully used the blue native system to study protein complex stability, to identify components of protein complexes by antibody shift experiments, and to analyse the cooperation of protein translocases by arrest of imported radiolabelled precursor proteins (Qiu *et al.* 2013).

Left: In protein gels, the mitochondrial scaffold structure with many identical copies of Mic10 looks like a ladder. Right: Maria Bohnert and Martin van der Laan. Photograph by: Wolfgang Fritz, University of Freiburg



The molecular chaperones – the heat shock proteins Hsp10 and Hsp70 – form a complex that is important for assembling Hsp60 molecules into a molecular barrel. Image: Lena Böttinger and Thomas Becker/University of Freiburg

Cell free expression of proteins for functional assays

Proteins are synthesized in cell-free translation systems based on rabbit reticulocyte or wheat germ lysate. For radiolabelling with [35S]methionine, the gene of interest is cloned into specific vectors (pGEM4z plasmids) that provide a strong promoter and ribosomal binding sites for optimal *in vitro* translation reaction. Recombinant amounts of a protein of interest for use in functional assays are produced in wheat germ based translation systems (Stroud *et al.* 2011).

***In vitro* mitochondrial protein import assay and assembly of protein complexes**

The *in vitro* protein import assay of radiolabelled precursor proteins allows the analysis of protein transport kinetics into isolated mitochondria. The uptake of proteins into mitochondria can be studied by resistance to externally added proteinase K. Protein import into and across the inner membrane is dependent on the membrane potential across the inner membrane. We use specific inhibitors of respiratory chain complexes and ionophores to deplete the membrane potential. The dependency on the membrane potential is an important indication whether a precursor is imported into the mitochondrial matrix or inner membrane. The analysis of protein import by blue native electrophoresis provides the possibility to detect assembly intermediates and the time-dependent formation of mitochondrial membrane protein complexes like respiratory chain complexes or translocase complexes (Böttinger *et al.* 2013; Qiu *et al.* 2013). We have established the import of radiolabelled precursors as well as of recombinant amounts of proteins. The import of recombinant amounts allows an *in vitro* complementation of mitochondria isolated from deletion strains. We have developed this experimental approach to discriminate between indirect and direct effects upon loss of the protein of interest (Qiu *et al.* 2013).

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Ethics

HEAD PD Dr. Joachim Boldt

PROJECTS

The future success of synthetic biology does not only depend on scientific and technological progress but on public and political acceptance as well. With regard to the latter, ethical arguments and evaluations are important to reach societal agreement.

The project package aims to analyse and evaluate ethical issues at three levels

1. Conceptual issues: Being a science and technology directed at the realm of life and strongly influenced by engineering ideals, synthetic biology challenges everyday concepts of life and their normative implications. Public unease and communicative barriers can be partly traced back to misunderstandings based on these different ways of conceptualizing the objects of synthetic biology research. Thus, different relevant concepts of life are analysed and their normative implications are delineated in order to enable a dialogue between scientists and the public that is not hampered by unwittingly applying different understandings of life.

3. Identifying relevant ethical criteria: Synthetic biology is a technology with potential applications with regard to health and food and agriculture. Hence, synthetic biology cannot be sufficiently dealt with by exclusively applying commonplace medical ethics frameworks or by using a (less operationalized) environmental ethics framework. Hence, a unified set of ethical criteria for evaluating synthetic biology applications will be established.

3. Risk assessment: Synthetic biology organisms may come to differ from their natural counterparts to such a degree that risk assessment procedures as they are commonly used in the case of GMOs may possibly prove to be insufficient. Therefore, paradigms of current risk assessment procedures will be analysed with regard to their ability to deal with possible future synthetic biology parts and organisms.

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Can research in the area of synthetic biology be correctly described as an act of creation of life? If so, does this have ethically relevant implications? Synthetic biology raises these and more philosophical and ethical challenges.
Image: © Feng Yu - Fotolia.com

METHODS

Conceptual research analysis

Aims and intentions of research approaches can be framed differently. This conceptual framing, in turn, influences ethical evaluations. Thus, synthetic biology research approaches are analysed in order to establish an adequate conceptual framing that can be set to use in ethical evaluations.

Establishing coherence of ethical criteria

At a medium and concrete level of abstraction, diverse ethical criteria can be deployed to assess research and research applications. Some of these criteria may stem from different ethical theories or even contradict each other. In order to establish a valid set of criteria, their conceptual coherence is tested.

Ethically assessing research projects

Ongoing and proposed research projects are assessed according to ethical criteria as currently agreed upon in the scholarly debate on technology ethics and bioethics.

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Apoptosis

HEAD Prof. Dr. Christoph Borner

PROJECTS

ER-mitochondrial signalling for apoptosis

Our group works on signalling processes involved between the ER and mitochondria which are crucial for apoptosis induction. We recently found that a member of the reticulon family of proteins (RTN3A1) interacts with Bcl-2-like survival factors. Our recent cellular and biochemical analysis indicates that the interaction between RTN3A1 and Bcl-2-like proteins occurs at mitochondrial/ER contact points. We are currently using high-resolution fluorescence microscopy and PLA techniques to prove the interaction at these sites. Moreover, we apply proteomics strategies to identify other proteins within this complex and measure calcium and lipid exchange between the organelles to see if the interaction between RTN3A1 and Bcl-2 regulates these processes.

Mechanisms of apoptosis signalling induced by fungal gliotoxin

We previously published that the virulence factor gliotoxin (GT) of *Aspergilllus fumigatus* kills primary lung epithelial cells by apoptosis via JNK-mediated Bim phosphorylation and subsequent Bax/Bak activation (Geissler *et al.* 2013). Since then we have been using various knock-out cell lines and lentiviral-mediated shRNA transfer to delete upstream signalling components of the JNK activation pathway. We found that GT activates JNK via a RhoA-ROCK-1-MKK-4-mediated pathway. We measured RhoA and ROCK-1 activity and inhibited the enzymes with pharmacological inhibitors and bacterial toxins. Currently, we want to identify the direct target of GT at the plasma membrane and for that purpose we perform mass spectrometry analysis with a variety of adhesion molecules. In addition, we developed an animal model in which mice are immunocompromised and develop Aspergillosis after inhaling the fungus. We will now use mice, which are deleted in different signalling components along the RhoA-JNK-Bim pathway to see if they are protected against disease.

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Mechanisms of apoptosis signalling induced by viruses
We found that the positive, single strand RNA virus Semliki Forest (SFV) induces apoptosis in various host cells via both Bax/Bak-dependent and -independent caspase activation mechanism (Urban *et al.* 2008). The Bax/Bak-independent caspase activation is novel and involves MDA-5/MAVS-mediated innate immunity signalling. The RNA virus triggers the formation of dsRNA which then activates this pathway not only to produce an anti-viral type I interferon response but also to induce apoptosis via the mitochondrial recruitment of caspase-8 to MAVS where the enzyme gets activated and cleaves and activates caspase-3 (El Maadidi *et al.* 2014). Recently, we also further defined the signalling leading to Bax/Bak-dependent caspase-3 activation. We found that

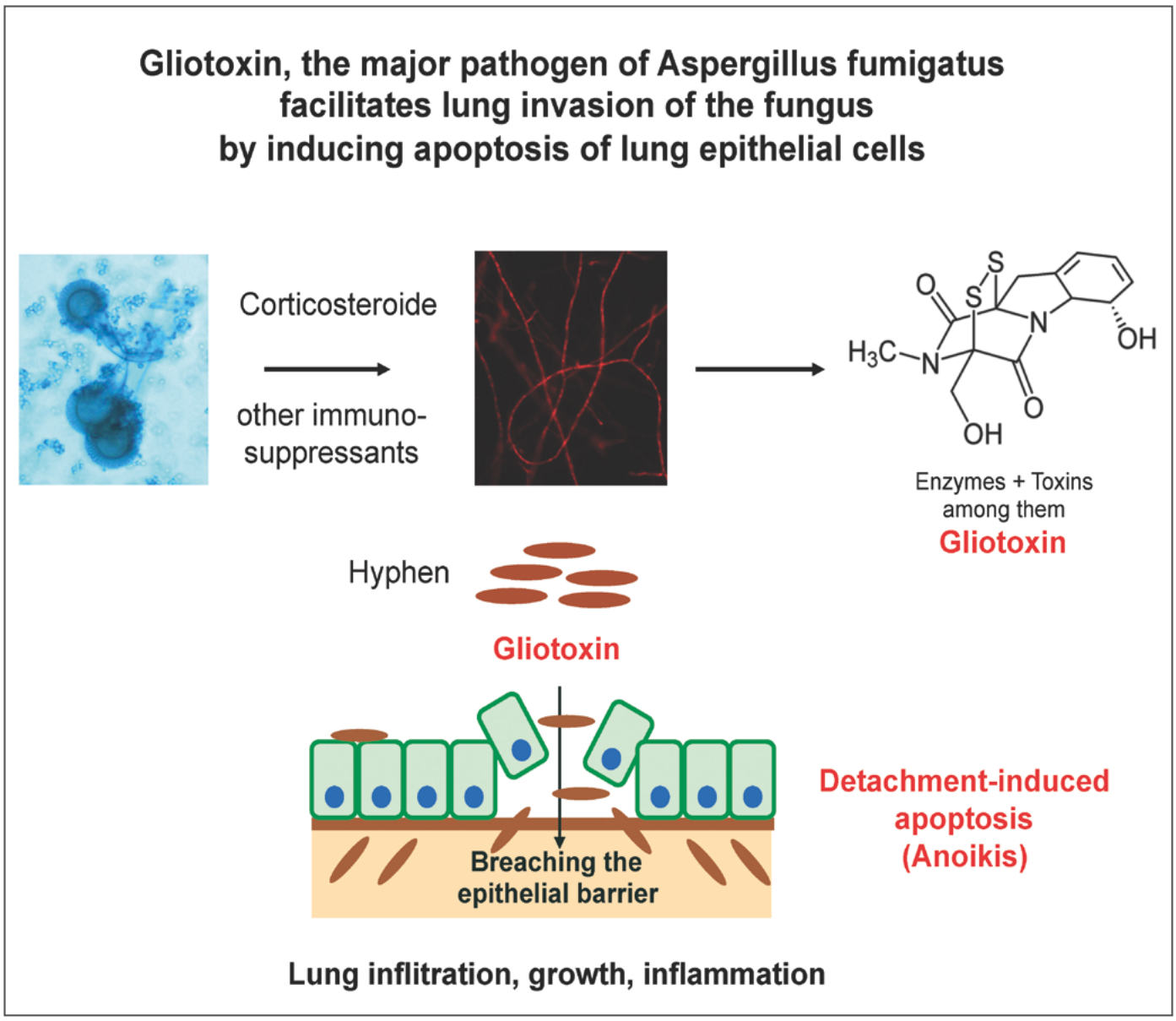

Image: Christoph Borner

SFV and also an unrelated DNA virus (herpes simplex virus-1, HSV-1) provokes the stabilization and activation of the BH3-only protein Puma which then directly activates Bax/Bak on mitochondria (Papaianni *et al.* 2015). We are now studying the posttranslational modification on Puma, which leads to its stabilization/activation and via which signalling pathway two distinct viruses are able to trigger this modification on Puma.

Posttranslation modifications and complex formation of Puma

In a DACH research group consortium of the DFG, we have recently begun to characterize the biochemistry and mode of action of the BH3-only protein Puma. We identified mouse and human cells, which die by apoptosis in a Puma-dependent manner and re-constituted Puma-/- cells with tetracycline and tamoxifen-inducible FLAG- and 3xHA-Puma constructs. These cells will now be used to determine posttranslational modifications and new binding partners (as well as Puma dimerization/multimerization) by co-immunoprecipitation/SILAC mass spectrometry, blue native gel electrophoresis and gel filtration analysis.

Sensitization of FasL signalling by TNFα in hepatocytes

In collaboration with the Prof. Dr. Irmgard Merfort group at the Institute of Pharmacology, we published that TNFα albeit itself not inducing apoptosis, sensitizes primary mouse hepatocytes and fibroblasts to FasL-induced apoptosis (Schmich *et al.* 2010). This might be crucial for increased liver damage and the generation of various liver diseases under inflammatory conditions such as alcohol consumption, fatty liver, various gut infections and hepatitis infections producing massive amounts of TNFα. The TNFα sensitization involves JNK activation and requires the BH3-only protein Bim. In addition, we could recently show that TNFα nduces a p65 NFκB-mediated upregulation of Fas on the surface of both fibroblasts and hepatocytes. Bim seems to enhance the trafficking of Fas from intracellular stores to the cell surface, but we do not yet know the signalling pathway by which Bim is able to regulate this process.

METHODS

Proteomics/SILAC mass spectrometry

We use the SILAC technique to label WT and knock-out cells treated with or without apoptotic agents with different amino acid isotopes. Total or membrane extracts from these cells are immunoprecipitated with different antibodies (including IgG controls) and the IPs pooled and run on an SDS-PAGE. Lanes are cut into equal pieces, digested with trypsin, and subjected to mass spectrometry to determine posttranslational modifications or binding partners of a given protein. In addition, we use blue native gels and gel filtration analysis to determine supramolecular protein complexes, which then will be resolved by mass spectrometry as well.

Isolation of primary hepatocytes

We established a reliable method to reproducibly isolate primary hepatocytes from mouse livers by collagenase reperfusion. The cells are plated on type I collagen and consistently show a viability of >75% for 3 to 4 days (Klingmüller *et al.* 2006).

***In situ* proximity ligation assay (PLA) PLA**

This assay involves DNA-oligo-coupled antibodies specific for two target proteins, a rolling circle amplification, and fluorescence-coupled oligonucleotides for detection (Soderberg *et al.* 2008). We use the technique to confirm protein-protein interactions on the endogenous and overexpressed levels, which have previously been detected in co-immunoprecipitations.

Apoptosis assays

We are using various quantitative assays to measure apoptosis. Annexin-V/PI FACS analysis, caspase-3 activity assays and anti-caspase-3 western blotting of total extracts, distribution of cytochrome c between mitochondria and the cytosol by western blotting, the cleavage of the caspase-3 substrates PARP or ICAD, DNA fragmentation and Comet assay, MTT viability assay, immunofluorescence analysis to measure the conformational change of Bax and Bak, the release of cytochrome c and caspase-3 activation.

RhoA activity assays

To measure RhoA activity, we perform the so called Rhotekin pull-down where a RhoA-binding domain (GST-fusion protein) covalently linked to Sepharose beads quantitatively pulls down active GTP-bound RhoA. As positive control for RhoA activation, we use the bacterial CNFy toxin. To inhibit RhoA, we take advantage of the inhibitory C3 toxin (both toxins were obtained from Prof. Dr. Klaus Aktories, Freiburg).

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Image Analysis and Computer Vision

HEADS

1. Dr. Olaf Ronneberger (Image Analysis)
2. Prof. Dr. Thomas Brox (Computer Vision)

PROJECTS

Projects are concerned with (a) researching new methodology that can solve more difficult image analysis problems and (b) adapting the methodology available in our group to the specific problems of BIOSS partners. Currently our fundamental research focuses on (1) new machine learning technology to teach the computer in hard pattern recognition tasks by showing correct examples, and on (2) new image alignment techniques.

Deep learning for segmentation and detection

For approximately two years a revolution has been happening in Computer Vision. New machine learning techniques based on artificial neural networks (so called deep learning) together with the immense computing capabilities of modern Graphics Processing Units (GPU's) outperform a large number of the up to now best hand-designed image analysis methods.

This project is concerned with making these concepts work also on new tasks, especially those that typically appear in conjunction with biomedical images. We developed a network architecture, called u-net, which applies deep learning to various biomedical challenges. The u-net currently performs best on an international benchmark for segmentation of neuronal structures in electron microscopy recordings. We have outperformed the state of the art in several other light-microscopic cell segmentation tasks. Currently, we are applying these techniques to the detection of Microglia cells in brain samples (cooperation with Prof. Dr. Marco Prinz), for segmentation of histopathological images (cooperation with Prof. Dr. Martin Werner, Pathology, Medical Centre – University of Freiburg), segmentation of migrating human keratinocytes (cooperation with Prof Dr. Matias Simons), segmentation of plant cells (cooperation with Prof. Dr. Klaus Palme), and detection of rosettes (cooperation with Prof. Dr. Virginie Lecaudey). Further applications are very welcome.

Elastic registration

A second central project is the development of image alignment (elastic registration) techniques. Beside the further improvement of registration techniques for large model organisms like zebrafish larvae (cooperation with Prof. Dr. Wolfgang Driever), we currently work on a system to explain complex tissue images with a set of elastically registered templates. We develop improved EM-stack registration methods that compensate the deformations of mechanical slicing to build an artefact-free 3D volume from serial slices. Moreover, we work on new methods to align 4D (3D+t) recordings of the Xenopus kidney to compare gene expression patterns by virtual co-localization (cooperation with Dr. Soeren Lienkamp).

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Image reconstruction

A third project develops new methods to compensate for the known shortcomings of microscopical imaging processes, like absorption and noise. We work on two-view attenuation correction (cooperation with Prof. Dr. Wolfgang Driever and Prof. Dr. Klaus Palme), time-lapse image denoising (cooperation with with Dr. Aristides Arrenberg and Prof. Dr. Wolfgang Driever), and recently started to work on a simultaneous tracking and denoising algorithm, that will allow to record time-lapse movies with much higher frame rates without increasing the laser power.

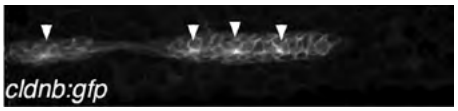
Quantification of patterns and constellations

After the relevant parts in an image are detected, segmented, measured, often the scientific biological question is not yet answered. We apply and extend methods, for example, to find clusters, to quantify certain constellations and patterns, and to measure the homogeneity and regularity of distributions. Concrete examples are methods to analyse cell patterns from synthetic biology (cooperation with Prof. Dr. Wilfried Weber), to analyse IgM clustering (cooperation with Prof. Dr. Michael Reth), to quantify the regularity of basal body spacing (cooperation with Dr. Albrecht Kramer-Zucker, and Prof. Dr. Gerd Walz) or microglia distributions (cooperation with Prof. Dr. Marco Prinz). Even though these works are not directly concerned with image analysis anymore, it is very important to carefully design these methods to draw correct conclusions from the image analysis results.

METHODS

We provide methods to extract quantitative information from images (2D), volumetric images (3D), videos (2D+t), and volumetric videos (3D+t) stemming from all sorts of imaging modalities. A detailed description of our methods will be available soon on the home page of the “Core Facility Image Analysis”: **http://cia.informatik.uni-freiburg.de**.

Detection: Counting certain objects / structures and finding their exact position



Example for a detection task. Detection of cell rosettes in the zebrafish primordium. Image: Ernst *et al.* 2012

Evaluation of images often requires the localisation of certain objects or structures. Examples are simple spots (e.g. fluorescently stained basal bodies (cooperation with Dr. Albrecht Kramer-Zucker, and Prof. Dr. Gerd Walz)), dividing and non-dividing cell nuclei in the *Arabidopsis* root (Schmidt *et al.* 2014), Microglia cells in brain samples (cooperation with Prof. Dr. Marco Prinz), cell rosettes (Ernst *et al.* 2012), or landmarks (like lenses, optic nerve, mid-hinbrain boundary, etc.) in zebrafish larvae (Ronneberger *et al.* 2012). The simple detectors need specification of some parameters (e.g. size of the spots). In more difficult cases, detectors can be trained to solve the task by providing a sufficiently large set of manually annotated examples.

The detection result is usually a list of positions. From this list we can compute, e.g. number of rosettes in a primordium, the basal body density on a cell surface, or the distances of microglia cells to their neighbours. Detected landmarks can be used to align images from different individuals (see "image alignment" below). Often the detected positions must be related to the overall anatomy. The anatomy is then extracted with another method (e.g., segmentation, model alignment, see below). E.g., we compute the number of dividing cells in the endodermis layer of the root.

Segmentation: Measuring size, shape, and constellation of objects / structures

Size, shape, or constellation of objects can be quantified based on a segmentation of the image. A segmentation method assigns to each point in the image one out of a set of labels (e.g. object and background). Segmentation objectives can be, for instance, "cells" and "background" (Ronneberger *et al.* 2015), "cell borders", and "cytoplasm" (Lienkamp *et al.* 2012), the seven different layers in an *Arabidopsis* root (Schmidt *et al.* 2014), different regions (e.g. Erythroposes, Granulopoese, etc.) in histopathological samples (cooperation with Prof. Dr. Martin Werner, Pathology), or all the anatomical regions in a zebrafish brain (Ronneberger *et al.* 2012).

Simple segmentation methods require setting some parameters (e.g. thresholds, minimal and maximal size of searched objects, etc.) and are often combined with image enhancement methods, e.g. segmentation of cells in *Arabidopsis* roots (Schmidt *et al.* 2014), cells in the *Xenopus* kidney (Lienkamp *et al.* 2012), or cells in the zebrafish primordium (cooperation with Prof. Dr. Virginie Lecaudey). In more difficult cases, the computer can learn to correctly segment images from a sufficiently large set of manually annotated examples (Ronneberger *et al.* 2015). The manual annotation procedure can be made more efficient using interactive training strategies. Another common method is to align the image to a reference atlas, and transfer the segmentation from this atlas, so-called "atlas-based segmentation" (Ronneberger *et al.* 2012, see "image alignment" below).

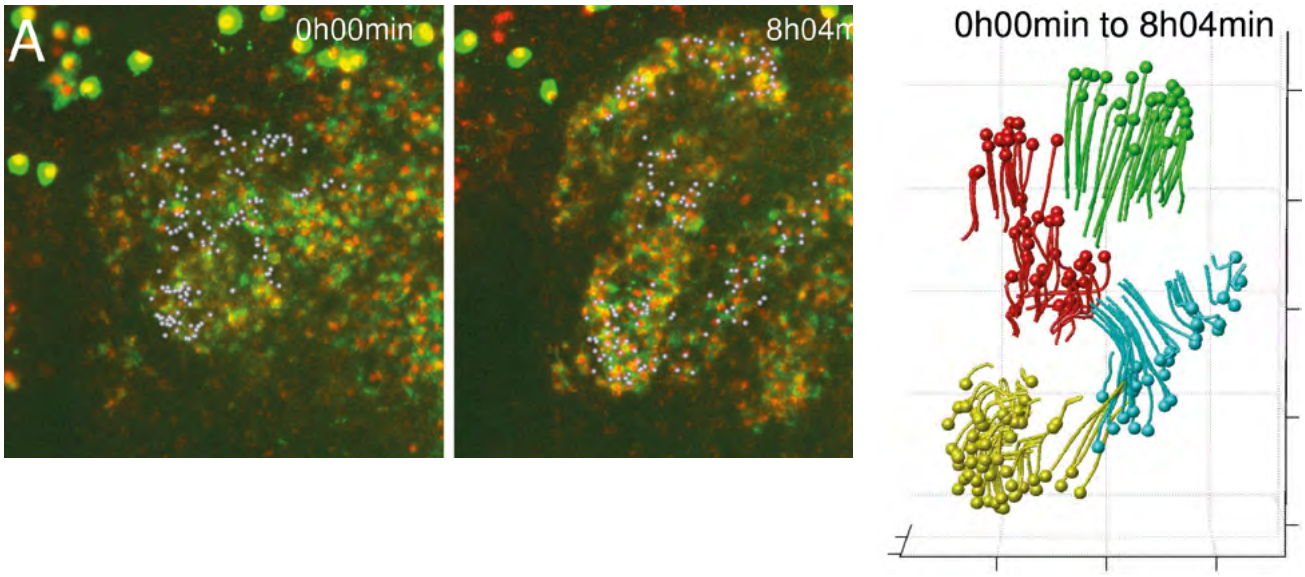
The segmentation result is usually a list of masks for the different object regions and a class-label for each region. From this list we can extract the number of objects per class, measure the size, diameter, circumference, and other shape features of the objects, for instance quantify protusions of cells in time-lapse recordings (Cooperation with Prof. Dr. Matias Simons). Furthermore we can analyse the constellations of objects, e.g., search for multiple cells that touch in a single point to find cell-rosette structures (Lienkamp *et al.* 2012), or find cell intercalation events during zebrafish epiboly (cooperation with Prof. Dr. Wolfgang Driever). As mentioned above, the regions are often used to relate marker patterns (recorded in a second channel) to the anatomical reference, e.g., quantify expression of TH in the post. tuberculum hypothalamus in the zebrafish brain, or determine the number and position of dividing cells in the endodermis layer of the *Arabidopsis* root.

Image Alignment: Compare images of different individuals

In many biological applications, images from different individuals at different experimental conditions should be compared. This can be based either on measures that are extracted from each image individually (see above), or based on a direct comparison of the recorded images (e.g. co-localization analysis). For such a direct comparison the images must be aligned to each other, which can be achieved by a so-called elastic registration. The elastic registration shifts, rotates, and deforms the new image in such a way that it fits best to the reference image. After a successful registration, all corresponding structures (e.g., the left eye in the new image and the left eye in the reference image of a zebrafish larva) lie on top of each other, and signals in a second channel (e.g. gene expression patterns) can be directly compared by virtual co-localization (Ronneberger *et al.* 2012). For samples like *Arabidopsis* roots, where no unique one-to-one correspondences exist, a direct registration is not possible. In such cases we either use an abstract model (here the layer model) and align each image with this model (Schmidt *et al.* 2014), or we use a larger number of small reference images (here individual cells) instead of a single large reference image, and model the new recorded root by a "puzzling" of small aligned reference images.

Tracking and Motion Analysis

Example for a tracking task in time-lapse recordings. Left: first frame. Middle: frame at time point 8h04min. Right: trajectories of several tracked cells from 0h00min to 8h04min.
Image: Lienkamp *et al.* 2010



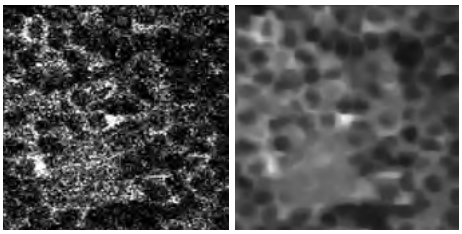
To analyse dynamic processes in time-lapse recordings (2D or 3D images over time) it is necessary to track (follow) certain objects or structures over time. If the objects are well separated, a two-step approach can be used. In the first step a segmentation (see above) is performed in each frame independently, and in the second step the segments from the first step are associated over time (Ronneberger *et al.* 2015).

The output of such a tracking approach is like in segmentation a list of masks, but for each objects t masks (for a time-lapse recording with t time steps) are obtained. These masks describe the evolution of the object over time. Like in the segmentation (see above) position, size, circumference, and other shape features can be computed and can be used, e.g. to plot the objects size over time.

Correspondingly, the constellation of multiple segments/objects can be computed, e.g. to find cell intercalation events during zebrafish epiboly.

An alternative approach directly tracks the structures in a time-lapse recording without prior segmentation. This approach is advantageous in cases where structures cannot be clearly localized or separated. It computes the motion vector of each individual pixel (or voxel) in each frame and concatenates all these vectors to dense trajectories over time. Afterwards, the user can manually select an object of interest in an arbitrary frame of the movie and immediately gets the full trajectory of this object. We applied this method successfully to the analysis of growth patterns in the proximal pronephros development in *Xenopus* embryos (Lienkamp *et al.* 2010), and for simultaneous tracking and segmentation of cells in dense tissue (Lienkamp *et al.* 2012).

Compensation of shortcomings in image recording



Example for image enhancement: Spatio-temporal denoising of time-lapse calcium-imaging recordings in the zebrafish brain. Images: Maxim Tatarchenko, Aristides Arrenberg, Wolfgang Driever und Olaf Ronneberger

Often the quality of an image suffers from known shortcomings, introduced in the preparation or recording process. Typical effects are deformations of the tissue during mechanical serial sectioning, absorption of light in thick samples, bleaching, image blur, movement of a living sample during recording, the limited field of view of high magnifying objective lenses, the limited dynamic range of the image sensor, or high image noise, if short illumination times, low excitation power, or weak fluorophores have to be used.

We have developed several methods to compensate such shortcomings, many of them are based on fusion of multiple recordings. Mechanical deformations of the slices can be estimated and compensated by image alignments methods (e.g., EM stacks in cooperation with Prof. Dr. Stefan Eimer). Deformations due to harsh preparation protocols, e.g. WISH staining of zebrafish larvae, can be compensated by an elastic alignment to a reference larva (Ronneberger *et al.* 2012). Light absorption in thick samples like zebrafish larvae or *Arabidopsis* roots can be compensated by fusion of two recordings of the sample from different views (top and bottom) (Ronneberger *et al.* 2012). Image blur can be reduced by deconvolution techniques, or a combination of multi-view recording with deconvolution techniques. Movement or deformation of a living sample during recording (e.g. due to the beating heart of a *Xenopus* embryo) can be estimated and compensated by image alignments methods (Lienkamp *et al.*

2012). The field of view can be extended by recording several images side-by-side and stitch them together (Emmenlauer *et al.* 2009, Ronneberger *et al.* 2012). The limited dynamic range of the image sensor can be extended by recording multiple images with different laser power and perform a high-dynamic-range fusion (Ronneberger *et al.* 2012). Image noise can be reduced by structure-specific image filters, e.g. that enhance cell-borders (Lienkamp *et al.* 2012, Schmidt *et al.* 2014), or by general-purpose denoising methods, e.g. to denoise time-lapse calcium-imaging recordings in the zebrafish brain, see Figure 5 (cooperation with Aristides Arrenberg, PhD, and Prof. Dr. Wolfgang Driever).

The resulting enhanced images are rarely the final output of an image analysis task. In most applications, image enhancement is the first step in a larger image analysis pipeline.

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Molecular Dermatology / Genetic Skin Diseases • Medical Proteome Research • Autoimmune Skin Diseases

HEADS

1. Prof. Dr. Leena Bruckner-Tuderman
2. Prof. Dr. Jörn Dengjel
3. Prof. Dr. Cassian Sitaru


1


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Photography by: University of Freiburg


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PROJECTS

Molecular genetics and mechanisms of mechanobullous skin diseases
The Bruckner-Tuderman lab is interested in the biology of basement membranes, the extracellular matrix, and epithelial-mesenchymal interactions. As genetic models and pathological correlates we investigate monogenetic skin fragility disorders caused by mutations in the genes for basement membrane proteins and epithelial adhesion molecules. A focus is on the molecular genetics and molecular and cellular disease mechanisms. Based on better understanding of disease mechanisms, development of biologically valid cell-, protein-, and small molecule-based therapies has recently become possible. We have different mouse models for skin fragility disorders, a patient registry with genetic and clinical data of more than 1,000 individuals, and an associated material bank.

Molecular pathogenesis of autoimmune skin diseases
Helper T (Th) cell-regulated development of antigen-specific B and plasma cells resulting in the production of pathogenic autoantibodies underpins the pathology of several autoimmune diseases. These include pemphigus, pemphigoid, myasthenia gravis, Guillan-Barre syndrome, autoimmune hemolytic anemia, immune thrombocytopenia, autoimmune dilative cardiomyopathy, Goodpasture's syndrome, systemic lupus erythematosus, anti-phospholipid syndrome, and rheumatoid arthritis. The Sitaru lab focuses on the mechanisms governing the coupling of autoantibody specificity to effector cell response, with special emphasis on autoantibodies against structural skin proteins in autoimmune blistering diseases. These diseases are clinically and immunopathologically well-defined entities where both the autoantigen and the pathogenic autoantibodies are well characterized. However, the complex pathogenic and regulatory mechanisms of pathogenic autoantibody production and several aspects of blister formation are still poorly understood. A major focus of our research is to investigate these pathogenetic mechanisms using *in vitro* and *in vivo* models of autoimmunity against structural skin proteins. In parallel projects, we study the structure and function of skin proteins, including collagens VII and XVII of the dermoepidermal junction such as collagen XVII (BP180) or p200, which are targets of the autoimmune response in a group of acquired blistering diseases. Ongoing patient-centred studies address the pathogenesis of pemphigus, a severe bullous dermatosis with intraepidermal blistering, with the aim of improving diagnosis and therapy.

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Development of novel diagnostic tools in autoimmune skin diseases

A further focus of the Sitaru lab is the development of new diagnostic and therapeutic tools for autoimmune diseases and the characterization of the biology of the autoantigens.

Skin disease proteomics

The Dengjel lab employs quantitative mass spectrometry-based proteomics to study deregulation of protein networks in skin disease. Loss of structural proteins has global implications on the composition of the extracellular matrix and cellular microenvironment which cannot be analysed by gene expression analyses. We use monogenetic as well as complex disease models to study the plasticity of the cellular microenvironment. Especially the role of fibroblasts as disease modifying and/or promoting cells is of interest.

Protein turnover by autophagy

Autophagy is a lysosomal degradation pathway important for cell homeostasis and stress resistance. Different forms of autophagy exist, amongst others macroautophagy, which is a constitutive process and also a stress response important for cell survival. Classically, it has been regarded as unspecific bulk degradation process. However, it has been shown that under stress conditions specific proteins can be selectively degraded by macroautophagy. The Dengjel lab studies the regulation and target selection of macroautophagy under different physiological stress conditions with the aim to identify new targeting mechanisms into autophagosomes.

METHODS

Isolation and culture of primary murine and human skin cells, organotypic skin cultures

The molecular dermatology labs have extensive experience in isolation and long-term culture of primary keratinocytes and fibroblasts derived from normal and/or pathologically altered murine and human skin. In order to obtain cell lines, keratinocytes are immortalized using the E6/E7 HPV technology. Organotypic 3D skin cultures are constructed with fibroblasts embedded in a biological matrix (dermal equivalent), overlayered with keratinocytes (epidermal equivalent) and cultivated in air-liquid interphase to mimic conditions of the skin *in vivo*.

Tissue morphological analyses

We also have extensive expertise in dermatohistopathological and morphometric analyses, and IF-molecular mapping of normal and pathologically altered murine and human skin, including skin fragility, wound healing, and tumorigenesis assays.

Molecular genetic mutation screening

The assays include Sanger sequencing and NGS (exome analysis). The lab has significant expertise in analyses of the sequencing data, interpretation of pathologically relevant variants, biological validation of the findings and genotype-phenotype correlations.

Autoantibody-induced granulocyte-dependent tissue damage

This assay also known colloquially as the cryosection model is an *ex vivo* model of autoantibody-induced granulocyte-dependent blistering. Dermal-epidermal separation induced by autoantibodies against the epidermal basement membrane allows for *ex vivo* studies using a model consisting of cryosections of normal human skin incubated with patients' autoantibodies and leukocytes from healthy volunteers (Florea *et al.* 2014; van der Steen *et al.* 2012). This well reproducible model may be utilized to dissect the molecular mechanisms of activation of human granulocytes by patient autoantibodies.

Induction of tissue damage by the passive transfer of autoantibodies in mice

Blistering of skin and mucous membranes after binding of autoantibodies to their target represents the T cell-independent phase of the efferent autoimmune response in autoimmune blistering diseases. To reproduce this blister formation, purified IgG antibodies against collagens XVII and VII or the basement membrane are injected repeatedly into mice (Oswald *et al.* 2012, Csorba *et al.* 2014). Various inbred mouse strains such as nude, BALB/c, SKH-1, and C57BL/6 mice are susceptible to blister induction by injection of antibodies against the epidermal basement membrane. These models are dependent on the recruitment and activation of granulocytes and may be used to dissect the inflammatory mechanisms of the tissue damage by autoantibodies in the skin.

Induction of the autoimmune in mice

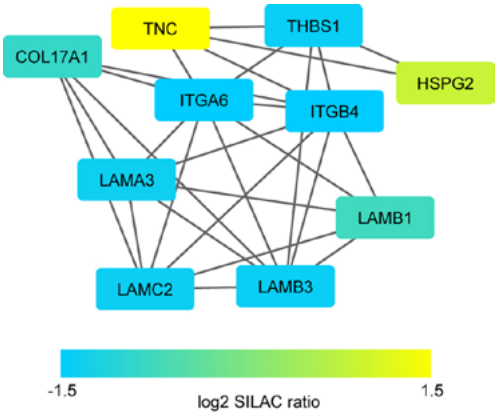
A sustained autoimmune response against the epidermal basement membrane and active blistering disease model may be induced experimentally by immunizing mice with collagen VII, the specific-target of autoimmunity in epidermolysis bullosa acquisita. To induce an autoimmune response against type VII collagen and blistering disease, mice are immunized subcutaneously in the footpads or at the tail base with 50 to 100 µg of recombinant autologous collagen VII (Sitaru *et al.* 2007).

Quantitative mass spectrometry-based proteomics

We employ label-free- as well as SILAC-based, quantitative mass spectrometry-based proteomics to perform expression proteomics, protein-protein interaction, and phosphoproteomics experiments. Different chromatographic fractionation techniques (protein- and peptide-based fractionation) are coupled to reverse phase-liquid chromatography ESI-mass spectrometry. Differences in protein abundances or posttranslational modifications are further analysed by imaging, molecular biological, and protein biochemical approaches.

Autophagic flux analysis

We use eGFP-LC3 and eGFP-dsRED-LC3 cells to analyse effects of discrete treatments on autophagic flux by western blot and image analysis.



Example for a detection task. Detection of cell rosettes in the zebrafish primordium. Image: Ernst *et al.* 2012

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Signal Transduction in Tumour Development and Drug Resistance

HEAD Dr. Tilman Brummer

PROJECTS

Our laboratory focuses on intracellular signalling pathways and how their intricate regulation is disturbed in human diseases and is influenced by clinically relevant drugs. Currently, we are working on three project areas:

Regulation and pharmacological inhibition of the Ser/Thr-kinase B-Raf
B-Raf represents an important oncoprotein, which is mutated in about 8 percent of all human cancer, with mutation rates rising up to 90 percent in certain tumour entities. Even in the absence of mutations, B-Raf is often dysregulated due to the mutation and/or over-expression of its activators such as Ras or receptor tyrosine kinases (RTKs). B-Raf is regulated by protein-protein-interaction and phosphorylation events provided by upstream activators as well as by crosstalk with other pathways and feedback loops. Using phospho-proteomics, we are working towards a more dynamic and refined model of the B-Raf activation cycle. B-Raf activity is strongly regulated by homo-dimerisation or by heterodimerisation with other Raf-isoforms or KSR proteins. Recently, we have characterised the importance of dimerisation for wildtype, oncogenic, and drug-inhibited B-Raf (Röring *et al.* 2012). For example, we could show that the formation of B-Raf homo- and heterodimers is based on distinct structural requirements and that oncogenic B-Raf mutants differ from wildtype B-Raf by forming particularly stable homo-dimers. In close collaboration with physicians, we are also interested in the molecular mechanisms by which novel tumour-associated BRAF mutations cut the aforementioned activation cycle short and contribute to tumorigenesis (Eisenhardt *et al.* 2011) and as to whether they respond to clinically relevant drugs. Using the insights stemming from our work on B-Raf signalling, we also work towards a better understanding of the mechanism underlying the success, failure, and side-effects of B-Raf inhibitors (Yaktapour *et al.* 2014).

Oncogenic signalling and metastasis
Another interest of the laboratory are the downstream signalling events by which oncoproteins such as B-Raf drive malignant transformation. To this end, we have generated several isogenic human epithelial cell line models, which allow us to express or suppress oncoprotein conditionally. For example, we have introduced a novel doxycycline (dox)-regulated expression system into MCF-10A mammary epithelial and Caco-2 colon adenocarcinoma cells. Using these systems, we are currently studying the impact of oncogenic kinases on various aspects of epithelial cell biology in conventional tissue culture and 3D culture systems (Herr *et al.* 2015). The latter systems provide an important bridge between *in vitro* and *in vivo* approaches and constantly improved. For example, we are working towards the BIOSS Cancer Monitor, a 3D culture system with a fluidic system that will allow the multi-parameter monitoring of 3D cultures in real time.

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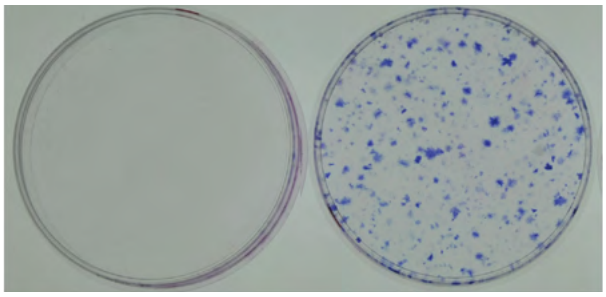

control BRAF mutant

Focus formation in SV40 large T antigen immortalised murine embryonic fibroblasts (MEFs) reveals the oncogenic potential of a novel tumour-associated BRAF mutant. MEFs were retrovirally transduced with either the empty pMIG vector as control or a pMIG vector containing a mutant BRAF cDNA. Cell lawns were supplied with fresh medium on every second day and stained with Giemsa solution after two weeks. Cells expressing the oncoprotein have lost the ability for contact inhibition and give rise to foci with increased dye retention. Each focus represents a transformed cell clone. Image: Tilman Brummer

The role of the Gab2 docking protein in tumour development and drug resistance

Docking proteins of the Grb2-associated binder (Gab) family represent important hubs in tyrosine kinase signalling networks. Following their recruitment to the plasma-membrane, these proteins become tyrosine phosphorylated and then recruit effector molecules with SH2 domains such as the tyrosine phosphatase SHP2 or the regulatory subunit of PI-3K, p85. This leads to the modulation and amplification of several downstream pathways involved in proliferation and survival. Gab proteins are subject to multiple positive and negative feedback loops modulating their signalling output and oncogenic potential. We are particularly interested in the Gab2 isoform, which is increasingly implicated in several human malignancies such as breast and ovarian cancer, melanoma, and chronic myelogenous leukemia (CML). Importantly, Gab2 signalling confers drug resistance to CML cells to clinically relevant Bcr-Abl inhibitors such as imatinib, nilotinib, and dasatinib (Wöhrle *et al.* 2013).

METHODS

Molecular biology

We apply all routine procedures of molecular biology.

Protein biochemistry

(Far) Western blotting, *in vitro* kinase assays, immuno-precipitation, recombinant protein expression, protein arrays.

Cell biology

We have extensive experience with various cell line models ranging from haematopoietic cells (lymphoma and leukaemia cell lines, primary bone marrow culture, primary leukaemia cells) to neuronal/glial cells (cell lines/primary astrocytes) to cells of epithelial origin (immortalised and primary mammary epithelia cells of murine or human origin; murine and human breast carcinoma), ovarian surface epithelial cell lines and ovarian cancer, lung and colorectal cancer cell lines as well as primary organoids from the murine small and large intestine (crypt cultures with stem cell niches). We apply retro- or lentiviral infection or conventional transfection or nucleofection to introduce cDNA or shRNA expression vectors into these systems.

In vivo approaches

We have extensive experience in the construction of conditional targeting vectors (Cre/loxP; Flp-e/Frt; tet-on/off Systems).

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Developmental Biology

HEAD Prof. Dr. Wolfgang Driever

PROJECTS

Dopaminergic neuron development

Dopaminergic neurons are lost in one of the major neurodegenerative diseases, Parkinson's disease, which has caused strong interest in mechanisms controlling dopaminergic neuron development from stem cells in the embryo as a model for potential regenerative approaches. We use the zebrafish as a genetic model system with excellent life imaging features to understand signalling mechanisms, transcriptional codes, and cell morphogenesis of dopaminergic neurons. Our work is focused on the evolutionarily most ancient dopaminergic neurons in the ventral diencephalon and hypothalamus. We have established mechanisms of precursor patterning, neurogenesis, signalling pathways, and transcriptional differentiation pathways for the major far-projecting dopaminergic system in the posterior tuberculum, corresponding to the mammalian A11 group, which sends ascending projections to the striatum, hindbrain, and spinal as well as endohypothalamic projections. Current efforts focus on analysis of signalling and epigenetic mechanisms that control development of dopaminergic neurons from neural stem cells at larval stages. Next to classical genetics, we use transgene technologies, TALEN and CRISPR based genome engineering, pharmacological manipulations, and develop optogenetic tools to spatially and temporally control dopaminergic differentiation in life zebrafish larvae.

Dopaminergic systems function

Dopaminergic neurons establish some of the major neuromodulatory systems, which modulate a vast array of neural circuits throughout the brain and spinal cord. While much effort has focused on the mammalian substantia nigra / ventral tegmental dopaminergic systems A8-A10, very little is known about the circuit activities of the evolutionarily highly conserved ventral diencephalic A11 system. We use genetic calcium indicators (GCaMP) optogenetic techniques in the zebrafish larvae to identify activity patterns of A11 dopaminergic neurons in behaving larvae and to manipulate circuit activities. Our work has identified specific motor- and/or sensory-correlated activity patterns in distinct A11 subgroups, opening up new avenues for understanding the function of these systems.

Integrated gene expression and signalling activity atlas of the zebrafish brain

In collaboration with the groups of Dr. Olaf Ronneberger, Prof. Dr. Thomas Brox, and Dr. Roland Nitschke, we have established a microscopy and image analysis framework for single cell resolution imaging of the whole zebrafish larval brain and registration of multiple brain specimens into an anatomical standard model (Virtual Brain Explorer for zebrafish ViBE-Z; Ronneberger *et al.* 2013). The atlas is populated with multiple neuronal differentiation markers to identify neuronal clusters, as well as transcription factor expression patterns to identify transcriptional codes for specific neuronal populations. We have also mapped

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Microinjection of zebrafish embryos for genetic manipulation. Image: Peter Mesenholl

the signalling activity of many developmental pathways (BMP, WNT, Shh, TGFb, Notch, etc.) using transgenic signalling reporter lines, and integrated the data into the anatomical atlas. The aim is to provide a framework for rational design of experiments into the role of specific signals and transcription factors into neural development.

Initiating development: Pou5f3 and gastrulation

The transcription factor Oct4 / Pou5f3 is a major regulator of pluripotency in embryonic stem cells. However, its functions in the early embryo and the evolution of the pluripotency regulatory network are not well understood. We investigate the regulation of developmental control genes and pluripotency network components in the early zebrafish embryo. Our data have shown a fundamental involvement in temporal control of the early embryonic transcriptional networks, and an evolutionary origin of the Oct4 / Pou5f1 regulatory network in zygotic genome activation (Leichsenring *et al.* 2013). We further use the gastrulation and cell behaviour defects in Oct4 / Pou5f1 mutant embryo as experimental system to study control of cell behaviour, adhesion, and migration, during gastrulation (Song *et al.* 2013).

METHODS

Genetics

We extensively use the excellent genetic tools developed for the zebrafish system to study mechanisms of development. Mutations are available for most genes, and transgenic reporter lines for many signalling pathways. Transgenic tools for Gain-of-function analyses using Gal4-UAS or HSP70 systems are used.

Genome Engineering

TALEN and CRISPR/Cas9 based genome engineering is established as tools to generate gene specific mutations as well as single or multi-gene deletions. Strategies for knock-in by homologous recombination are currently optimized and used to make reporter or driver lines as well as conditional alleles using the Cre-lox system.

Gene expression analysis

High-resolution gene expression analysis by chromogenic or fluorescent whole mount *in situ* hybridization. Integration of gene expression data in atlas using ViBE-Z framework. High throughput transcriptome analysis using (i) manually dissected tissue, (ii) laser micro dissection, (iii) genetically fluorescently tagged cells and FACS. RNA-seq and bioinformatics analyses (CLC Genome Workbench).

Experimental embryology

A wide range of experimental approaches is available ranging from RNA and DNA microinjections to cell transplantations and cell lineage analysis.

Microscopy

We extensively use fluorescence and confocal laser scanning microscopy for life imaging of cell behaviour and circuit activity in zebrafish embryos and larvae. Most microscope techniques provided by the Life Imaging Center of the University of Freiburg are established for life zebrafish imaging in the lab (CLS, SD, MP, TIRF).

Embryo sorter

A fluorescence activated embryo sorter helps to sort rare genetic events with fluorescent read-outs from large populations of larvae.

Pharmacological manipulations and chemical genomics

Small and medium scale screens using chemical or pharmacologically active compound libraries are established to screen for effects on specific developmental processes. Current efforts are focused on compounds effecting epigenetic mechanisms of development.

Optogenetics

“Physiological” optogenetics tools (sensors like GCamP, actuators like ChR2, NpHR) visualize and manipulate neuronal activity *in vivo*. “Genetic” optogenetic switches are developed for light controlled gene expression in zebrafish (collaboration with the lab of Prof. Dr. Wilfried Weber).

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Metalloproteins and Membrane Proteins

Photography by: University of Freiburg

HEAD Prof. Dr. Oliver Einsle

PROJECTS

Biological Nitrogen Fixation

A central focus of our work is nitrogenase, the only enzyme able to break the triple bond of the N2 molecule, making atmospheric nitrogen available for assimilation into biomolecules. We have recently focused on the unique FeMo cofactor, the active site of nitrogenase, that we found to contain a unique central carbide (Einsle *et al.* 2002; Spatzal *et al.* 2011). More recently, we were able to demonstrate ligand binding to the cofactor for the first time using the slow substrate carbon monoxide (CO) (Spatzal *et al.* 2014). The exact mechanism of nitrogenase still remains to be elucidated and presents one of the grand challenges of current bioinorganic chemistry.

Nitrous Oxide Reductase

The atmospheric concentration of the greenhouse gas nitrous oxide (N2O) is on a steady rise due to industrial and agricultural activities. A copper-containing enzyme, nitrous oxide reductase, mediates biological reduction of N2O to uncritical N2. We have elucidated the structure and basic functional principles of the enzymes (Pomowski *et al.* 2011), and we are currently in the process of refactoring nitrous oxide reductase in a synthetic biology approach. Our aim is to address the problem of oxygen sensitivity that currently prevents the enzyme's use in bioremediatory applications using a directed evolution strategy.

Novel Multiheme-based Redox Enzymes

A third group of metal-containing proteins in the focus of our current research are multiheme cytochromes c, a diverse family of heme-containing proteins that catalyse a wide range of multi-electron redox reactions. A recent result was the structural and mechanistic characterization of a novel sulfite reductase with a unique heme-copper active site (Hermann *et al.* 2015). We have established the recombinant production of a variety of enzymes of this family, allowing not only for analysis, but also for modification and control of their catalytic capabilities for possible biotechnological applications.

Integral Membrane Proteins

A second major research area of the group is integral membrane proteins, in particular solute carriers and ion channels. Beside a primary focus on the determination of novel three-dimensional structures, we also functionally reconstitute isolated proteins into artificial membrane systems for functional studies. Current work includes the study on the bacterial formate channel FocA (Lü *et al.* 2011) and the nitrite channel NirC (Lü *et al.* 2012), both members of the FNT family of pentameric ion channels found in bacteria and plants that play a central role in the energy metabolism of pathogenic enterobacteria such as Salmonella. More recently, the work on membrane proteins has extended towards maturation systems for different metal cofactors.

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METHODS

Protein Crystallization and Structure Determination:
Crystallization of biological macromolecules is carried out using automated liquid handling systems for buffer preparation (Rigaku Alchemist II) and nanoliter dropsetting (Art Robbins Phoenix, Douglas Instruments Oryx Nano). Crystallization experiments are carried out in 96-well plates that are automatically inspected at regular time intervals. The facility is set up to accommodate more than 1,400 plates at two temperatures. Collaborations for structure determination are welcome anytime. Protein crystals can be evaluated on in-house diffractometers on a rotating-anode X-ray generator that is also suitable for high-resolution data collection. Since diffraction experiments require tunable X-ray sources and high-quality diffraction data, we have regular access to synchrotron radiation sources, primarily the Swiss Light Source (SLS) and the European Synchrotron Radiation Facility (ESRF).

Anoxic Protein Biochemistry:
Many metal-containing enzymes and proteins originate from anaerobic metabolisms and contain functional metal sites that are highly sensitive to the presence of O2, due both to its high redox potential and its chemical reactivity. We have therefore established a complete workflow for preparative and analytical protein biochemistry under strict exclusion of dioxygen. Rather than relying on glove boxes and inter gas chambers, we use heavily modified and optimized Schlenk techniques adapted from preparative inorganic chemistry. Independent on the actual stability of a metal centre towards dioxygen, we also observe that the control of redox state is a crucial determinant for the functionality and crystallizability of many protein targets.

Isothermal Titration Calorimetry:
This method allows for the direct determination of the thermodynamic parameters (entropy and enthalpy) and the stoichiometry of protein:protein or protein:ligand interactions. Dissociation constants can be straightforwardly determined with high accuracy, and in spite of a relatively high demand for protein material, its precision makes titration calorimetry the method of choice for most ligand interaction studies.

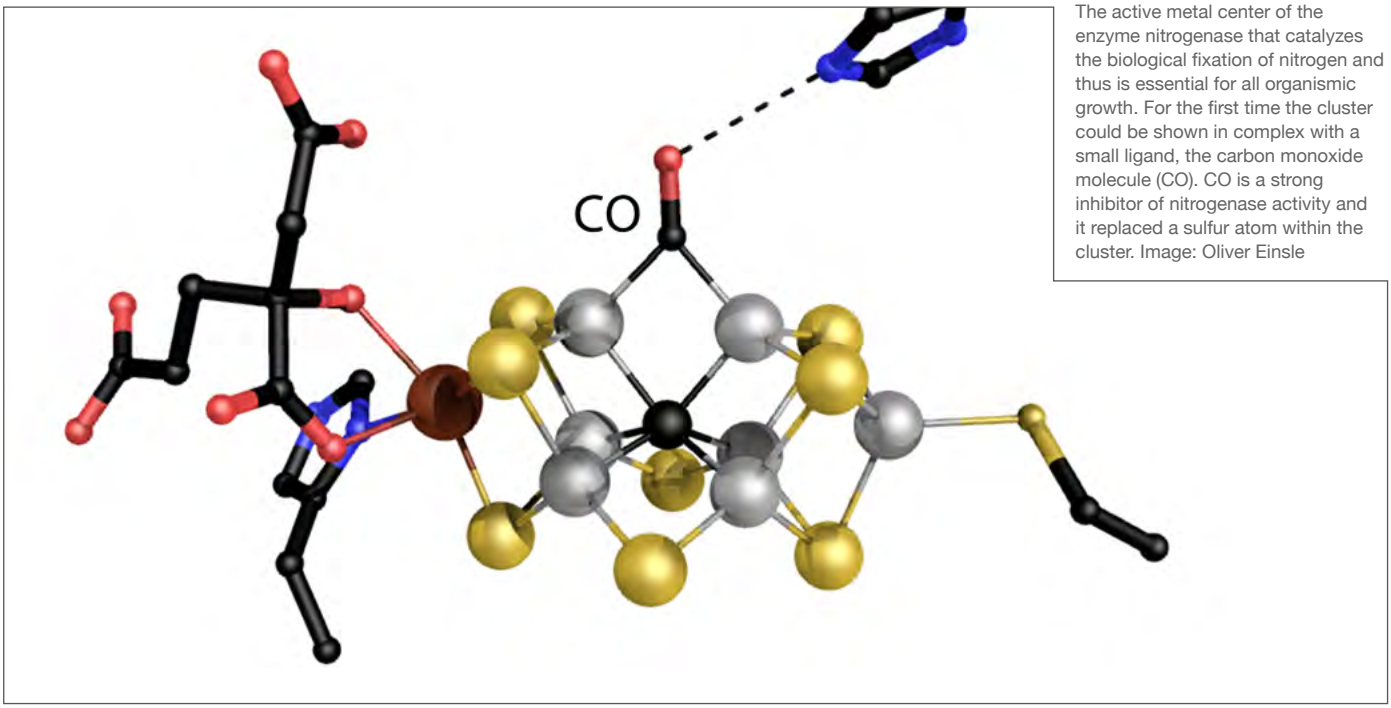
Thermofluor Stability Assays:
Studying protein:ligand interactions as well as protein stability in general is also possible by using a regular RT-PCR thermocycler. It monitors thermal denaturation of a target protein through the fluorescence change of a fluorophore, such as SYPRO orange, which interacts with the hydrophobic region of the protein that becomes accessible upon the disintegration of the tertiary structure at a given 'melting point'. This method has also proven highly useful in screening for stabilizing point mutations, which tend to improve the resilience of a protein as well as its crystallization behaviour.

Analytical Size-exclusion Chromatography:
One of the prime determinants showing whether a protein is likely to form crystals is its homogeneous dispersion in the buffer solutions. The formation of unordered aggregates is a frequent problem that can hinder or prevent downstream analyses. A series of parameters, including ionic strength, pH, and temperature can significantly influence such aggregation, though. Their control can help to obtain the monodisperse samples required for successful crystal growth. In order to monitor monodispersity, but also micelle size of membrane protein preparations, we use a Malvern/Viscotek analytical size-exclusion chromatography workstation with a triple detector array. It allows for the continuous and parallel measurement of full absorption spectra with a diode array, refractory index, and static light scattering for the determination of particle size.

Gas Chromatography:
In our work on metalloenzymes in nitrogen metabolism, the key enzymes of interest convert gaseous substrates, such as N2, CO, C2H2, or N2O. Gas chromatography thus represents an important and direct way to monitor substrate conversions in enzyme assays, but also to identify new reactivities in novel enzymes identified through metagenomic screening. We are using an SRI gas chromatograph with FID and TCD detectors that can easily be adapted for new columns if the need arises.

UV/vis Spectroscopy:
Due to the presence of metal cofactors, most metalloproteins under investigation in the group show characteristic absorption features and colours that change with redox state and can thus provide essential insight into the catalytic capacities of an enzyme. UV/vis spectroscopy is routinely carried out to obtain this information, and a series of spectrometers is available for different applications. These include single- and dual-beam spectrometers for high-precision measurements, but also fast CCD and diode array devices. Spectra are routinely recorded under exclusion of oxygen to maintain full control of the redox state of a sample in question.

Electron Paramagnetic Resonance (EPR) Spectroscopy:
Unpaired electrons are a rare occurrence in biological macromolecules, and only few amino acid residues are found to form such metastable radicals. More commonly, organic cofactors or metal centres are employed to handle unpaired electrons, and their existence and location is usually tightly linked to the functional role of the protein. In a close technical



The active metal center of the enzyme nitrogenase that catalyzes the biological fixation of nitrogen and thus is essential for all organismic growth. For the first time the cluster could be shown in complex with a small ligand, the carbon monoxide molecule (CO). CO is a strong inhibitor of nitrogenase activity and it replaced a sulfur atom within the cluster. Image: Oliver Einsle

analogy to nuclear magnetic resonance (NMR), the EPR experiment interrogates the electronic structure of a sample in a magnetic field by applying microwave radiation that is specifically absorbed by single electrons oriented along the magnetic field lines and causes them to change their magnetic moment (spin). In a large sample with thousands of atoms, EPR spectroscopy focuses exclusively on the one (or few) unpaired electron and thus provides an excellent tool for mechanistic and structural studies (Spatzal *et al.* 2013). Our group uses a Bruker Elexsys 500 continuous-wave spectrometer for X-band and Q-band measurements equipped with a helium cryostat for low-temperature experiments.

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Cellular Structure Biology

HEAD Prof. Dr. Stefan Eimer

PROJECTS

Research focus of the professorship for Cellular Structure Biology is to gain a molecular as well as structural understanding of molecular membrane transport processes, their regulation in the normal state, and their deregulation in the disease state.

Rab GTPase regulation of membrane trafficking

Rab GTPases are the largest subfamily of Ras-type small GTPases, which act as molecular switches regulating virtually almost all aspects of vesicular membrane trafficking and sorting between cellular compartments and to the cell surface. In their active, GTP-bound state, Rab GTPases localize to membranes where they organize specialized membrane micro-domains through the recruitment of effector molecules. While there are over 69 Rab GTPases encoded in human genome with multiple redundancies, the genome of the nematode *Caenorhabditis elegans* only encodes for 26 Rab GTPases, with mostly one unique member per subtype. Therefore, we are using the multicellular model system *C. elegans* to study the regulation of molecular membrane trafficking and signalling (Sasidharan *et al.* 2012).

Cellular structures and their function for membrane trafficking

Vesicular membrane transport between within the secretory and endomembrane system within a cell requires highly coordinated interactions of different intracellular compartments. The functionality of these trafficking compartments thereby strongly relies on their structural integrity, which may change during aging and in disease states. To understand how the structure and function of intracellular compartments are connected, we are studying their 3D ultra-structures at high resolution using modern electron microscopy (EM), EM-tomography as well as super-resolution microscopy techniques. Currently, we are focussing on three main structures, i) the Golgi apparatus as the main trafficking and sorting hub within the secretory system, ii) mitochondria, as the key organelle for cellular homeostasis and aging, iii) as well as the synapse as the main communication unit between neurons.

Cellular function of Parkinson's disease genes

Although genome wide association studies have revealed genes linked to familial forms of Parkinson's disease (PD), the mechanisms by which they induce the disease and subsequent neurodegeneration is still not clear. We are particularly interested in understanding the early cellular alterations that lead to the development of the disease. To address this question, we take advantage of *C. elegans* as a transparent and easily tractable model system. We are studying how loss-of-function of PD-associated genes changes cellular structures, trafficking, and physiology, particularly focusing on cellular stress responses and homoeostasis. In this context, we are analysing how lysosomal integrity and autophagic turnover are required for the quality control of cellular

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compartments such as mitochondria, which are required for normal aging and cellular fitness (Karpinar *et al.* 2009).

METHODS

High-pressure Freeze (HPF) Electron Microscopy (EM) Sample Preparation

So far, classical chemical fixation has been the method of choice to prepare samples for EM analysis. This method requires the infiltration of fixative into the tissue prior to embedding in plastic resin. Due to time and dehydration required for chemical fixation, the fine structures are altered and the morphology suffers. Recently, high-pressure freeze (HPF) sample preparation has been developed as an alternative protocol, which does not require chemical pre-fixation of the sample. During HPF, sample preparation biological samples are directly cryo-immobilized within milliseconds through ultra-rapid application of liquid nitrogen. The speed of the freezing process largely prevents the formation of ice crystals, leaving tissue morphology intact. Afterwards the sample, embedded in amorphous ice, is transferred in liquid nitrogen to a freeze substitution chamber (AFS). In the AFS, the free water of the sample is slowly (over days) at -90°C substituted and the sample is embedded in plastic resin. Now, ultra thin sections can be cut and analysed using conventional EM microscopy (see first picture). The main advantage of the HPF technique is the highly improved morphology and ultrastructure of the sample with less volume artifacts. Since this technique allows a preservation of the ultrastructure of the sample, it is also the basis for super-resolution microscopy and correlative light and electron microscopy (CLEM).

Analysis of Cellular Ultrastructures in 3D by EM Tomography

Since EM sections are usually around 40 to 50 nm thin, the information about 3D structures is limited. However, for complex tissues and organs the information about the overall 3D structure is particularly important. In combination with an improved ultrastructural preservation through HPF, 3D EM reconstructions yield important insights into the global 3D organization of cells and tissues. In order to understand the 3D ultrastructure of cells and tissues, we are using EM tomography, which allows the reconstruction of cellular ultrastructures in 200 to 500 nm thick sections. This is the basis for a quantitative analysis of cellular phenotypes in the normal and in the disease or mutant state. EM tomography allowed us to solve the complex cellular 3D structure of mitochondrial networks, trafficking compartments such as the Golgi-apparatus (see second picture), and neuronal synapses (Witte *et al.* 2011; Kittelmann 2013a/b).

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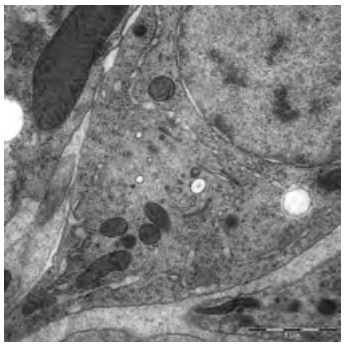

Transmission EM ultrastructure of a cholinergic motoneuron of *C. elegans* prepared by HPF EM.
Image: Research group Eimer

3D ultrastructure of a secretion unit: 3D EM reconstruction of Endoplasmic Reticulum (ER) exit sites with COPII coated transport vesicles and adjacent Golgi apparatus in *C. elegans*. The structures were reconstructed by EM tomography of a 250 nm thick section. The endoplasmic reticulum (ER) is coloured in silver with ribosomes as purple puncta. ER exit sites with the forming COPII-coated transport vesicles (red) are shown. After budding off the ER, the COPII coated vesicles are uncoating and fusing laterally within the ER-Golgi intermediate compartment (orange). From there the forming cisternae is maturing to become the cis-Golgi in yellow, medial Golgi in green, and the trans-Golgi compartment in blue. Microtubules, which anchor the ER and Golgi structures, are shown as white rods.
Image: Research group Eimer


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Photograph: Eric Plichta

Molecular Physiology

HEAD

1 Prof. Dr. Bernd Fakler

PROJECT LEADERS

1 Prof. Dr. Bernd Fakler
2 PD Dr. Akos Kulik
3 Dr. Uwe Schulte
4 Dr. Jochen Schwenk

PROJECTS

Rapid signal-transduction at and across the plasma membrane of neurons is fundamental for perception, transfer, processing, storing, and retrieval of information. Key molecular players in this context are ion channels controlled either by the transmembrane voltage or by ligands/agonists, as well as G-protein coupled receptors (GPCRs). All of these elements operate with an enormous precision in both time and space and endow (inter)cellular signalling with a high-degree of specificity and adaptability (in response to activity and exogenous stimuli). Our group investigates the molecular aspects and mechanisms that determine organization, dynamics, and operation of rapid neuronal signal transduction in projects that are funded through various sources including BIOSS, collaborative research centres, and individual grants.

In particular we have developed an approach known as *'functional proteomics'* that enabled identification of the comprehensive sets of protein building blocks for several distinct types of ion channels (incl. AMPA-type glutamate receptors or voltage-gated Ca^{2+} (Cav2) and K^{+} (Kv1) channels, as well as GPCRs for GABA (GABAB) and glutamate (mGluRs)). The respective results showed that these key players of brain function are not stand-alone elements (as suggested by molecular cloning and disease genetics), but rather multi-protein assemblies of different complexity (protein complexes, super-complexes, and networks). In fact, the subunit composition of these assemblies varies in time as well as between brain regions, sub-regions, and types of neurons (and synapses), thus strongly suggesting that the high-degree of specificity observed in functional experiments is encoded by the properties of the complex assemblies. Central challenges and questions for future research on this novel concept of membrane signalling are: How and where do the protein complexes assemble, how are they processed, what governs their subcellular targeting and their dynamics in response to exogenous stimuli, and, in particular, what is the functional significance (and primary function) of the many complex constituents identified that so far lack annotation of any function (roughly 55% of the whole mammalian genome).

Answers to these questions will finally help us understand the molecular details and mechanisms underlying brain function including formation of memory in health and their role for generation of diseases (such as epilepsies, mental retardation, or Parkinson's disease).

METHODS

For *high-resolution functional proteomic analysis of membrane protein complexes* we established a strongly interdisciplinary workflow comprising a variety of techniques including the ones briefly outlined below:

Solubilization of membrane protein complexes

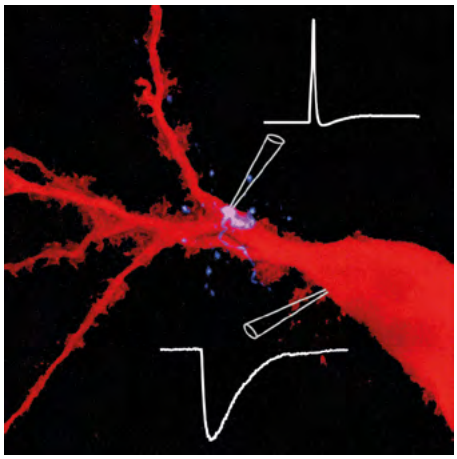
This is the most critical step in the analysis of membrane protein(complexe)s as it requires the use of detergents acting as a solvent, but at the same time destabilizing the protein-protein interactions underlying formation of protein assemblies (*Schwenk et al.* 2010, 2012). In collaboration with Logopharm GmbH we have developed a series of buffer systems (single detergents or combinations of various (classes of) detergents) that are compatible with comprehensive proteomic analysis of complexes from native membrane preparations (*Berekfeld et al.* 2006, *Schwenk et al.* 2012). All of these buffers (termed ComplexioLytes) are detailed on the Logopharm web site (www.logopharm.com) and are commercially available.

Native separation of protein (complexes)

For analyzing molecular arrangement and size of protein assemblies, as well as for probing compatibility of detergent buffers with complex formation we use blue native gel electrophoresis (BN-PAGE). In this technique, solubilized protein(complexe)s are separated (after centrifugation on a sucrose gradient) on linear polyacrylamide gels (e.g. 1% to 11%) according to their apparent molecular mass. Combined with pre-incubation with target-specific antibodies, BN-PAGE can also be used to identify distinct populations of complexes or integration of a defined target into given complexes (antibody-shift assay; *Schwenk et al.* 2009).

Multi-epitope affinity-purification (ME-APs) for comprehensive proteomics of native protein complexes

Comprehensive analysis of nano-architecture and subunit-composition of 'native protein(complexe)s' requires their isolation from membrane preparations and subsequent unbiased identification of their constituents. For isolation we use affinity-purifications (APs) with a series of antibodies raised against different epitopes along the primary sequence of the target (to counteract peculiarities of any individual antibody). Subsequent analysis by high-performance liquid chromatography-coupled tandem mass spectrometry (nano-LC MS/MS) provides data on the identity (MS/MS) and relative quantity (integrated peak volumes, PV) of all proteins in the eluate(s) of the APs (dynamic range of 10.000; *Bildl et al.* 2012). Comparison of protein amounts between sample APs and negative controls (APs with target-specific antibodies on protein preparations from target knock-out animals, APs with pre-immunization IgGs) finally enables determination of the subunits reconstituting the native form(s) of a given target protein (*Schwenk et al.* 2010, 2012).



The 'paired-bouton configuration' places one pipette in the presynaptic terminal and another one in the soma of the contacted postsynaptic cell.
Image: BFakler

Micro-proteomics

To study the dynamics and local specificity (or diversity) in subunit-composition of target complexes, we optimized the ME-AP procedure to allow for proteomic analysis in micro-tissue punches (diameters of tissue samples as small as 200 µm; *Schwenk et al.* 2014).

Cryo-slicing blue-native MS analysis (csBN-MS) for complexome profiling

As an antibody-independent approach to characterize protein-protein interactions and to determine stoichiometries of complex constituents, we are using blue BN-PAGE coupled to mass spectrometry (BN-MS). In a first step gently solubilized protein complexes are separated by their size on a preparative pore gradient gel. The gel lane is then embedded and subjected to precise sub-mm (0.1 to 0.5 mm) sampling using a cryo-microtome for slicing. Slice samples are then individually digested with trypsin and the resulting peptide mixtures analysed by nano-LC MS/MS. After stringent processing of these data (involving assignment to identified peptide sequences / proteins, mass and retention time calibration, noise filtering, and intensity normalization) relative abundance-mass profiles are obtained for more than 1,000 proteins over a broad range of complex sizes (100 to 3,500 kDa). The exquisite size

resolution (< 5% MW difference, reflecting the limit set by BN-PAGE) and quantification accuracy allows for (i) comprehensive characterization of multiprotein complex subunit composition, (ii) detection of subtle molecular heterogeneities of complexes and (iii) identification of new complexes and discrimination of overlapping complexes by correlation-based methods with high confidence. When combined with standards for absolute quantification (QConCAT technique), this approach can also be used to determine the stoichiometry of proteins in distinct complex populations (*Schwenk et al.* 2012, *Turecek et al.* 2014).

Ultrastructural analysis of protein complexes: SDS-freeze-fracture replicas and immuno-electron microscopy

To investigate precise (co)-localization (resolution of ~20 nm) of membrane-bound protein(complexe)s and their activity-dependent dynamics, we use the newly developed sodium dodecylsulfate-digestedfreeze-fracturereplicaimmunolabelling (SDS-FRL) technique (complementing conventional pre- and postembedding immunogold approaches). The brain tissue is frozen and freeze-fractured thus allocating proteins either to the proto/cytoplasmic (P-face) or exoplasmic face (E-face) of the plasma membrane. Molecules are immobilized with a thin layer of carbon followed by deposition of platinum/carbon for shadowing; the tissue underneath the carbon/platinum-layer is dissolved by SDS leaving proteins trapped in carbon.

The SDS-FRL method has several advantages over conventional techniques: (i) higher sensitivity for detecting molecules as membrane proteins are exposed on the 2D surface of the replica and (denatured) epitopes are readily accessible for antibodies; (ii) high reproducibility of protein distribution patterns and labelling intensity; (iii) accessibility of large membrane areas and number of synapses in (compared to conventional methods requiring three-dimensional reconstruction of labelled compartments).

Virus-directed manipulation of gene expression *in vivo*

In order to assess the primary function and/or significance of a complex constituent we manipulate its abundance in target cells (or regions) by virus-mediated either knock-down or (over)expression of protein(s) in wildtype and mutant form (site-directed mutants or tagged proteins). The viruses (lenti- or AAV viruses) are delivered by stereotactic injections into anaesthetized rodents (*Boudkkazi et al.* 2014).

Sub-cellular patch-clamp recording (from individual synapses)

In addition to more conventional patch-clamping (whole-cell mode, excised patches combined with sub-millisecond solution exchange, *Schwenk et al.* 2009), we established the 'paired-bouton configuration' that places one pipette in the presynaptic terminal and another one in the some of the contacted postsynaptic cell. This configuration enables precise recordings from and control of the signal transduction in individual synapses (*Boudkkazi et al.* 2014).

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Toolbox

HEAD Dr. Nicole Gensch

As the scientific community becomes more and more complex, easy and fast sharing of information, methods, and material is an important factor in scientific research. To facilitate and accelerate the exchange of expertise and biological material between scientists and laboratories, the *Toolbox* was initiated with BIOSS.

The Toolbox established a *plasmid repository* at BIOSS to reduce the burden on individual labs to store, maintain, and distribute plasmid clones and supporting information. Our repository has currently a large collection of over 1,300 plasmids deposited by individual researchers. All material collected for the repository is properly annotated and sequence verified. The wide diversity of plasmids from scientific sources ranges from the research fields of synthetic biology to plant biology, signalling pathways in Drosophila, B and T cell receptor (BCR, TCR) signalling as well as vectors with different fluorescent and photoactivatable proteins to visualise and study protein interactions (e.g. Bimolecular fluorescence complementation (BiFC) analysis). Our repository includes also a library of over 1,600 constructs of mouse specific shRNAmir sequences, which affect approximately 500 mouse specific kinases. Furthermore, we distribute clones of the human ORFeome V5.1 cDNA clone collection and the Yeast ORF collection widely to the BIOSS community and members of the University of Freiburg. Our mission is to provide researchers with inexpensive plasmid service and, thus, to expand the research capacity of scientist and to increase their working efficiency.

In addition to our plasmid repository, we also provide BIOSS researchers with a portfolio of *standard operating procedures* with workflows and protocols. Such protocols serve to standardise best practice and enable an enhanced research transparency due to its easy accessibility to the community. We develop protocols for each cell line that has been submitted to our *cell line repository* by researchers. This includes, among other things, a protocol to establish transfection methods for the different cell lines and to *generate stable* cell lines expressing a gene of interest. Long term expression of a protein of interest (or knockdown of a gene of interest) is an important research tool in the life sciences. While transient transfections limit the types of cell-based assays one can perform, cell lines with stable expression can be used for long term experiments (e.g. growth and differentiation assays). Another advantage of a stably expressing cell line is that it can be utilised for large scale production of proteins for purification. Two general procedures are used to integrate the expression system into the cells: plasmid based or viral based (retroviral or lentiviral expression systems). Following antibiotic selection resistant cell colonies can be obtained and clonal selection can be achieved with *cell sorting* by using flow cytometry and limited dilution cloning. The Toolbox cell line repository with diverse standard cell lines from mouse and hamster to human is available for all BIOSS scientists. In addition, we perform mycoplasma contamination control and identification of a cell line by genotyping.

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BIOSS combines different research areas and, depending on the purpose of the research, specific vectors and DNA constructs are required. The *Toolbox Cloning* platform collaborates with researchers and supports their scientific work in cloning individual expression vectors. Whether for overexpression or knockdown: We design and generate constructs of bacteria, mammalian, and viral expression systems. According to the requested purpose, we use a different strategy to clone the gene coding sequence into a wide range of expression vectors.

In addition to our repository service, the Toolbox core facility is equipped with a broad spectrum of devices to support researchers in their projects. Our specialized fields are *Protein Expression and Purification, Imaging* and *High Throughput Screening*. With focus on method development, the Toolbox scientists Dr. Pavel Salavei, Dr. Giulia Mizzon, and Dr. Susan Lauw are working closely together with BIOSS researchers to develop new powerful methods in the field of cell signalling and synthetic biology.

One of the Toolbox key units is the *Protein Expression and Purification* facility. Dr. Pavel Salavei takes care of all the available instruments such as Flow Cytometer, ÄKTA chromatography Systems, HPLC and trains users to handle those devices.

Large scale protein expression

Our facility has two bioreactors Celligen310 (2.5 L and 14 L) for culturing mammalian and insect cell lines, which can be operated in batch, fed-batch, and perfusion mode independently from each other or connected in series. Using optimized protocols, higher protein expression could be achieved compared to shaker flasks or rolling bottles. The 14 L sterilisable-in-place BioFlo415 Fermentor is a robust production scale system, designed to meet any research and production needs. In fed-batch process, high cell density (OD600>100) could be reached that is at least by factor 10 higher than in shaker flask cell culture.

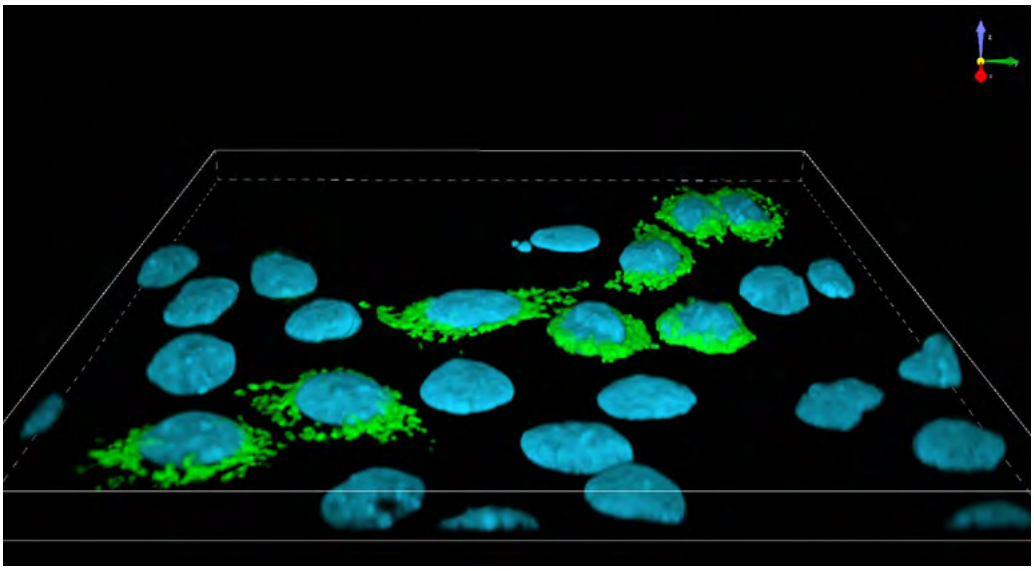
Protein purification

A number of ÄKTA chromatography systems are available in the Toolbox for separation and purification of biomolecules at scales ranging from micrograms to grams. Our ÄKTA systems are equipped with a broad range of chromatographic columns and media, which cover all main types of chromatographic techniques: size exclusion, cation and anion exchange, affinity, hydrophobic interaction, and immobilized-metal affinity (IMAC) chromatography. We have experience in the purification of membrane, secreted, and cytoplasmic proteins.

Protein modifications

We developed and optimized protocols regarding coupling proteins to different molecules. Using special reagents, a protein can be coupled to another protein, to DNA, or to fluorescent dye. Depending on the nature of modification, a single labelled protein can be separated from a double or multiple labelled protein.

Dr. Giulia Mizzon in the *Toolbox Imaging Platform* takes care of the microscopes in the Signalhaus, trains the users, and supervises the imaging projects of several students. Standard techniques include light and fluorescence microscopy in epifluorescence wide field, confocal, and total reflections mode.



3D rendering of confocal z-stack of fixed CHO cells. Mitochodria are labelled with MTS-EGFP (green) and nuclei with DAPI (cyan).
Image: Dr. Giulia Mizzon

A method routinely carried out here and used now by several BIOSS groups to investigate the nanoscale organisation of membrane is the *Proximity Ligation Assay*. In this context, efforts are in place to speed up the acquisition and analysis of these samples.

With focus on method development, we collaborate in several projects that require special imaging conditions or instruments. In close collaboration with BIOSS research groups, we develop and establish protocols for *super-resolution microscopy* such as directed stochastic optical reconstruction microscopy *(dSTORM)* and photo-activated localization microscopy *(PALM)*. Experiments in dSTORM mode have already been carried out on cell culture samples. Moreover, *correlative light and electron microscopy* projects are under development in collaboration with the EM facility and the Faculty of Biology (Prof. Dr. Stefan Eimer).

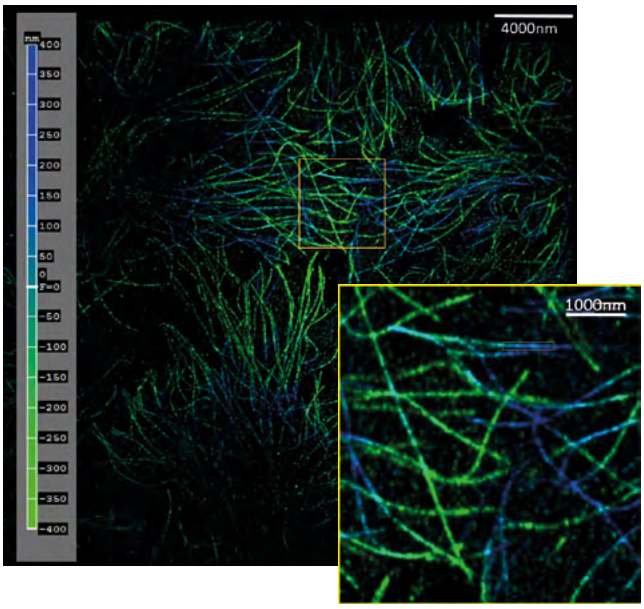
Enzymatic labelling methods as the *sortase technology* are of increasing interest in the field of labelling small proteins or DNA oligos. We are working in collaboration with the Reth group on establishing this method at BIOSS to specifically label nanobodies or single-chain antibody fragments of interest. Plasmids to generate fusion proteins with a sortase recognition site (LPXTG) are available from our plasmid repository.

The BIOSS *High Throughput Screening* (HTS) facility is equipped with a Tecan Freedom Evo 200 Liquid Handling System that is able to pipet volumes of 1 µl to 1,000 µl with high accuracy. The liquid handling system allows automated library splitting with pipetting and diluting up to 10,000 libraries per day. We perform automated assay screening with the liquid handling system (Tecan) together with microplate dispenser (Biotek) and washer (Biotek 405TMTS). The Biotek Synergy H4 Hybrid reader enables detection of absorption, fluorescence, luminescence, FRET/TR-FRET, and fluorescence polarization in 96- and 384-well plate format. We collaborate in several projects to develop new assays and screening methods that require automatisation systems.

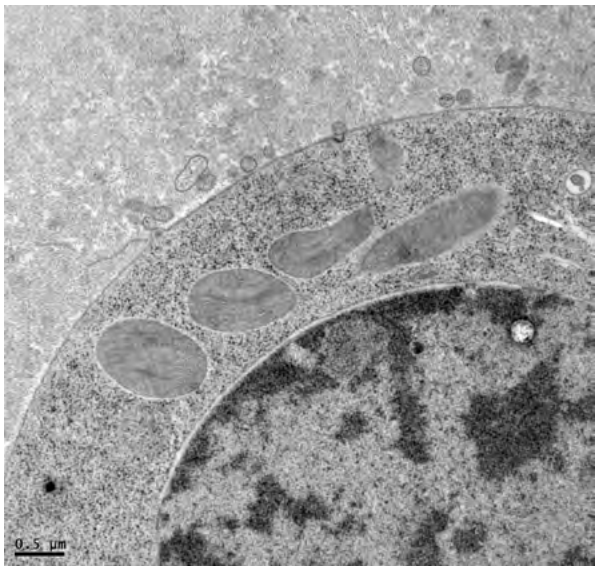
Aptamer selection

An aptamer is single stranded DNA, RNA, or protein that binds to a specific molecular target with high affinity and selectivity by folding into its secondary and tertiary structure. Aptamers are useful tools in basic research as they can be generated *in vitro* and can be selected to target any chosen ligand with high specificity and affinity. The technique of selection uses the SELEX method (systematic evolution of ligands by exponential enrichment). We are currently trying to optimize the method for High Throughput Screening and assay to test aptamer binding. Once established, we can make the method available for BIOSS scientists.

3D-dSTORM image of HeLa cells, indirect immunolabelling of tubulin. Color indicate z position, as determined via astigmatism method. Images: Dr. Giulia Mizzon



Transmission electron microscopy image of Ramos cells: cells were high pressure frozen, freeze substituted, and infiltrated in epon. Detail of membrane vescicules and mitochondria. Sample Prep: Dr. Giulia Mizzon, TEM acquisition: Dr. Marta Rodriguez and Rosula Hinnenberg. Thanks to Prof. Dr. Stefan Eimer for access to instruments.



Photograph: Jürgen Brandel

Molecular Apoptosis

HEAD Prof. Dr. Georg Häcker

PROJECTS

Initiation of mitochondrial apoptosis

Our group works on understanding the activation of the Bcl-2 family of proteins, which initiate mitochondrial apoptosis. The factors that determine the activation are changes in protein levels as well as post-translational modifications. In terms of protein levels, anti-apoptotic proteins may be down- and pro-apoptotic proteins up-regulated. We have found a deubiquitinase that removes K48-ubiquitin from at least two Bcl-2-family members, regulating their abundance and co-determining apoptosis. In terms of post-translation regulation we have been studying the role of complex formation within the Bcl-2-family and have identified a new form of complexes within the pro-apoptotic BH3-only group of proteins that have a role in determining the regulation of apoptosis.

Synthesising mitochondrial apoptosis

Essential components of mitochondrial apoptosis have been identified but the molecular mechanisms that govern their activity are still widely uncertain. We have recently identified the need for C-terminal insertion of BH3-only proteins into the mitochondrial membrane. Currently we are studying the molecular interactions of a number of components (membranes, the BH3-only protein Bim, the facilitator of Bim-complex-formation, dynein light chain 1, and the effector of mitochondrial apoptosis Bax) in a cell-free system *in vitro* to understand sequence of events and stoichiometry of the various interactions of apoptosis-regulating players. A further, new approach is the use of the light-responsive LOV-domain to cage the C-terminus of BH3-only proteins, permitting light-dependent induction of apoptosis.

Modelling the role of signalling pathways in the differentiation of immune cells

To have the opportunity of studying signalling events in a near-physiological cellular environment, yet with great flexibility, we have established a system for the *in vitro*-derivation of progenitor cells that can be differentiated into immune cells (neutrophils, macrophages, dendritic cells, B cells, and (somewhat less efficiently) T cells). This system, which relies on the conditional activity of the oncogene Hoxb8, is easily amenable to genetic manipulation and well suited to the study of signalling events and networks during immune cell differentiation and in immune cell function, both *in vitro* and *in vivo*. We are currently studying signalling events of apoptosis and pathways downstream of cytokine receptors in these cells, using various models of genetic deficiency.

bioss.science/pm/molecular_apoptosis

METHODS

The CRISP/Cas9 technique

The technique is now used by many groups and permits the easy introduction of genetic modification of cells. We are in particular using lentiviruses to introduce Cas9 into cells and have adapted the system for the use of the progenitor lines mentioned above.

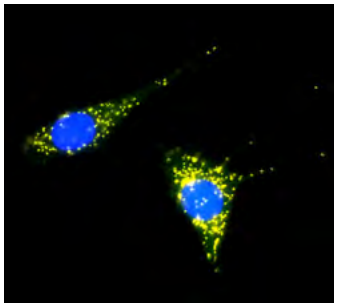
The mitochondrial apoptosis technique

This is essentially the rebuilding of the events that determine the essential step of mitochondrial apoptosis, namely the release of cytochrome c from mitochondria. Our cell-free reconstitution system uses mitochondria isolated from various cell types, which may be protease digested to remove endogenous signalling molecules. Often mitochondria from cells deficient in the effectors Bax and/or Bak are used. Recombinant or *in vitro*-synthesised BH3-only proteins (triggers of mitochondrial apoptosis) are imported into the outer mitochondrial membrane. The capacity of the mitochondria to activate Bax is tested by adding recombinant Bax. The test for activity is either to immunoprecipitate Bax with an antibody specifically recognizing active Bax, or to measure (Western blot) the release of cytochrome c into the supernatant. With this system, variants of BH3-only proteins as well as post-translational mechanisms can be tested.

In situ proximity ligation assay (PLA) uses DNA-oligo-coupled antibodies specific for two target proteins, a rolling circle amplification, and fluorescence-coupled oligonucleotides for detection (Soderberg *et al.* 2006). We have been using the method to detect complexes of Bcl-2-family proteins on mitochondria *in situ*.

Activation of BH3-only proteins by uncaging a Light-Oxygen-Voltage-sensing (LOV)-domain

LOV-domains are often found in non-animal, especially plant cells, where they serve as light-sensors. LOV-domains have been engineered to be suited to induce conformational changes in proteins in animal cells upon light-stimulation (Strickland *et al.* 2010). We are making use of this technique to engineer photo-reactive BH3-only proteins. In these constructs, the C-terminus, which is required for mitochondrial insertion and activity of BH3-only proteins, is 'caged' by the LOV-domain. Irradiation with blue light exposes the C-terminus, releasing the pro-apoptotic activity. We are optimizing this system to improve pro-apoptotic activity while reducing spontaneous activity in the absence of light.



Mouse embryonic fibroblasts expressing Bim with two different antibody tags were analysed by proximity ligation assay using antibodies against the tags. Yellow spots show PLA-signals, indicative of Bim-Bim interaction (which occurs on mitochondria). Blue, Hoechst stain for DNA/nuclei. Picture: Research group Häcker

Generation of immune cell progenitors

We have established and adapted a system for the differentiation of haematopoietic progenitor cells that can have the potential to differentiate either into defined lineages or into both myeloid cells and lymphocytes (first reported in (Redecke *et al.* 2013)). Progenitor cells are generated by expression of conditionally active Hoxb8, can be expanded *in vitro* and differentiated both *in vitro* and in mice. We have used cells committed to the neutrophil lineage in a number of settings including complex functional tests (Schwab *et al.* 2014) and have established the system to derive early progenitors. Mouse bone marrow cells are transduced with oestrogen-regulable Hoxb8. In the presence of FLT3 ligand, broad-potential progenitor cells expand; inactivation of Hoxb8 leads to the differentiation into cells of various lineages, depending on the culture conditions (e.g. GM-CSF generates myeloid cells, OP9 cells B cells, OP9-DL1 cells thymocytes). Upon injection into mice, myeloid cells, dendritic cells, and B cells are generated efficiently, T cells with lower efficiency. Progenitor cells can be generated from any mouse and can themselves be genetically modified, permitting great flexibility. Cells can be generated in large numbers, allowing biochemical and cell biological study.

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Molecular Mechanisms of Differential Gene Expression in Health and Disease

HEAD Prof. Dr. Andreas Hecht

PROJECTS

Developmental regulation of Wnt/β-catenin target genes

The Wnt/β-catenin signal transduction pathway plays an important role in almost every aspect of animal development and adult tissue homeostasis. Wnt/β-catenin signalling controls cellular proliferation, migration, lineage commitment, differentiation, and survival. A major characteristic of Wnt/β-catenin signalling is its ability to control different groups of genes in a highly cell-type-specific and temporally-coordinated manner. Wnt/β-catenin signalling therefore provides an excellent model system to investigate the molecular basis for differential gene expression. Key players in this process are heterodimeric protein complexes formed by β-catenin and members of the LEF/TCF family of transcription factors. Our research focuses on the role of LEF/TCF proteins, their functional diversification by alternative splicing, the functional impact of post-translational modifications, and the mechanisms that restrict the ability of LEF/TCF proteins to access cis-regulatory elements in a chromosomal context, including epigenetic modifications of DNA and chromatin (FAIRE, ChIP, EMSA, DNA methylation).

Disruption of gene regulatory networks during colorectal cancer progression

Wnt/β-catenin signalling is a driving force throughout all stages of colorectal tumorigenesis. Surprisingly though, whereas some Wnt/β-catenin target genes have oncogenic properties, others function as tumour suppressors. Examples for this are the *receptor tyrosine kinases EPHB2* and *EPHB3*, which control cell migration and restrict tumour spreading by an *E-cadherin*-dependent compartmentalization mechanism. The aim of this project is to unravel changes in *gene regulatory networks* that uncouple tumour suppressor gene expression from Wnt/β-catenin signalling and lead to their secondary *transcriptional silencing* (Figure 1). Thereby, we will gain insights into principle mechanisms that promote colorectal cancer progression. Our experimental approaches centre around the identification and functional characterization of regulatory DNA elements (FAIRE, ChIP, EMSA, CRISPR/Cas9 system), in particular *transcriptional enhancers*, which exhibit differences in their activity in a model of colorectal cancer cell lines and intestinal organoids. Our mechanistic studies of *enhancer decommissioning* and EPHB2/EPHB3 gene silencing revealed a change in the functional importance of *Notch signalling* and point to the importance of inducers of *epithelial-mesenchymal transitions*. Future studies aim to understand the full extent to which signal transduction processes and transcription factor activity are changed in colorectal carcinomas versus the

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healthy intestinal epithelium and how these changes impact on stemness, cellular identity, differentiation, migration, and invasion.

METHODS

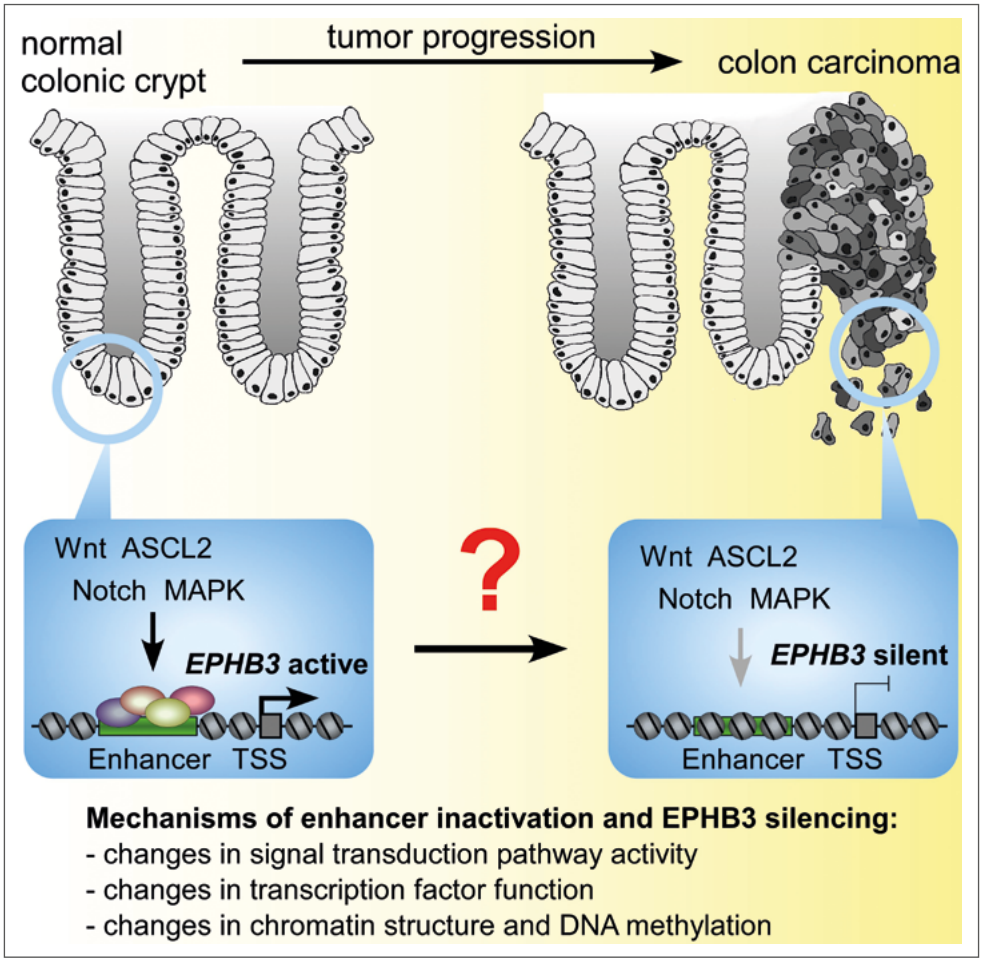
The unifying theme of our methods portfolio is the structural and functional analyses of *cis-acting DNA regulatory elements* and their associated *transcription factors* including a host of *DNA and histone modifying enzymes* in different cellular systems.

Intestinal organoid culture

The intestinal epithelium represents a *stem cell system* par excellence that allows studying changes in gene expression which accompany cell proliferation, migration, lineage commitment, differentiation, and cell death. Recently, a cultivation method has been established to grow intestinal organoids or so-called *"mini-guts" in vitro* that essentially *recapitulate every in vivo aspect of cellular behaviour*. The mini-guts can be propagated over extended periods of time without noticeable phenotypic or molecular changes. They are *self-organizing* in the absence of a mesenchymal component and *can be manipulated* by treatment with small-molecules, transfection, and lenti- or retroviral infection. In addition, they can be established from genetically-modified mouse strains. We use the intestinal organoids as an *ex vivo* system to model to intestinal tumorigenesis.

Formaldehyde-assisted isolation of regulatory elements

In their active states, cis-regulatory DNA elements such as *transcriptional enhancers* and *promoters* display as a characteristic structural feature the absence of nucleosomes which distinguishes them from bulk chromosomal, non-regulatory DNA. The absence of nucleosomes from active DNA elements can be exploited to selectively *enrich* the corresponding *regulatory DNA sequences* from the genome by formaldehyde-assisted isolation of regulatory elements (FAIRE). The assay principle is based on the differential segregation of protein-free DNA (active regulatory DNA elements) and DNA bound by histones (the constituents of nucleosomes) during phenol/chloroform-extraction of chromatin fragments derived from formaldehyde-treated cells. We use FAIRE to *assess the functional state of regulatory elements* in different cell types and under different signalling conditions (Jägle *et al.* 2014;



The research group of Andreas Hecht wants to unravel changes in gene regulatory networks that uncouple tumour suppressor gene expression from Wnt/β-catenin signalling and lead to their secondary transcriptional silencing. Picture: Andreas Hecht

Wallmen *et al.* 2012). Unlike chromatin immunoprecipitation, FAIRE does not require prior knowledge of transcription factors bound to regulatory elements. The FAIRE technique can also be used as a *discovery tool* to systematically identify active regulatory DNA elements in a *genome-wide* manner by next generation sequencing of FAIRE-enriched DNA (FAIRE-seq).

Chromatin-immunoprecipitation

A key issue in the study of differential control of gene expression is to determine which transcription factors and co-regulators are bound to regulatory DNA elements in a particular cellular background and at a given time. Chromatin immunoprecipitation (ChIP) is the method of choice for this type of question. Briefly, to fix the *in vivo state* of the *chromatin landscape* and *transcription factor occupancy* at regulatory elements, live cells are fixed with formaldehyde. Upon cell lysis and chromatin fragmentation highly specific antibodies are used to *immunoprecipitate proteins-of-interest* and their *associated DNA fragments*. The DNA content of the immunoprecipitate can be analysed in a region-by-region manner using qPCR or in a *genome-wide* manner by next generation sequencing *(ChIP-seq)*. We use ChIP to study the differential occupancy of enhancer and promoter regions by *transcription factors* and their associated cofactors (*histone acetyltransferases, deacetylases,* lysine *methylases* and *demethylases*), by *histones* and by *post-translationally-modified histones* in different cell types. ChIP also allows us to follow the *dynamic exchange* of transcriptional activators and repressors during the silencing of tumour suppressor genes in the course of colorectal cancer progression (Jägle *et al.* 2014; Rönsch *et al.* 2011; Rönsch *et al.* 2015; Wallmen *et al.* 2012).

DNA-Methylation

Changes in gene expression are frequently accompanied by alterations of cytosine methylation within so-called *CpG-islands* associated with promoter regions of afflicted genes. Promoter hypermethylation is one of the mechanisms that can lead to long-lasting transcriptional silencing of developmentally-regulated genes and tumour suppressor genes. To assess DNA methylation states, we have three different methods available. Genomic DNA can be subject to *restriction enzyme digest* with *methylation-sensitive isoschizomers* followed by PCR amplification (Rönsch *et al.* 2011). Alternatively, we use *affinity precipitation of methylated DNA* and quantification of the precipitate by qPCR (Rönsch *et al.* 2011). The third method involves the *chemical conversion* of non-methylated cytosines into uracil, PCR amplification of the converted DNA, subcloning, and sequencing *(bisulfite sequencing)*, which is most laborious but provides the most comprehensive information about the methylation state of individual cytosines in different cells of a mixed population.

Electrophoretic mobility shift assay

The functionality of regulatory DNA-elements arises from their ability to interact with a specific set of transcription factors and their associated co-regulators. The electrophoretic mobility shift assay (EMSA) is a technique that allows to identify and characterize presumptive *transcription factor binding sites* and to characterize *transcription factor complexes*. The EMSA technique is based on differences in the migration of unbound and protein-bound DNA probes in electric fields under *native conditions*. We have adapted this technique to the use of *non-radioactive*, biotin-labelled DNA probes and a highly sensitive *chemiluminescence detection* system (Rönsch *et al.* 2015; Weise *et al.* 2010).

CRISPR/Cas9-based genome editing

CRISPR/Cas9-based genome editing for molecular dissection of regulatory DNA elements. In the past, the *functional analyses of transcription factor binding sites in their native chromosomal context* was nearly impossible. This has now changed with the advent of novel genome editing techniques. In case of the CRISPR/Cas9 system, short RNA molecules are utilized to direct the Cas9 nuclease to specific genomic sequences where it introduces DNA double strand breaks. These DNA breaks can be repaired by non-homologous end-joining to introduce random mutations or by *homologous recombination* when a suitable donor DNA is present. We use the CRISPR/Cas9 system in conjunction with *mutant donor DNA* for the targeted introduction of *single nucleotide substitutions* in transcription factor binding sites to study their importance for enhancer function and gene expression without additional genome rearrangements.

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High Purity Cell Sorting of Glomerular Cells

Photograph by: Roger Koeppe/FRIAS

HEAD Prof. Dr. Tobias Huber

The architectural design of our kidneys is amazingly complex and culminates in the 3D structure of the glomerular filter. With the rising prevalence of chronic kidney disease, further molecular understanding of disease mechanisms is of paramount importance.

PROJECTS

Mechanisms of glomerular development

The nephron is the functional unit of the kidney and is essential for the filtration of blood, water and electrolyte homeostasis, and secretion of waste products into the urine. The number of nephrons is decided before birth in humans and is highly variable. There is a clear correlation between nephron endowment and the risk to develop hypertension and Chronic Kidney Disease. Many factors, such as maternal malnutrition, diabetes, and smoking, as well as low birth weight have been shown to lead to reduced nephron numbers. We are investigating the role of epigenetic modifiers during kidney development and the environmental factors involved in nephron differentiation.

Signalling networks regulating kidney regeneration and glomerular aging

Glomerular diseases, such as Chronic Kidney Disease and Diabetic Nephropathy are on the rise worldwide. The weakest link in the initiation and progression of glomerular diseases is the podocyte, a structurally complex cell crucial for the filtration of blood. Damage and loss of podocytes contribute to a vicious cycle of proteinuria and renal scar tissue formation. We are investigating the role and limits of podocyte regeneration, signalling pathways involved in podocyte damage and repair and the role of parietal epithelial cells in glomerular crescent formation.

Role of mTOR signalling for tubular function and disease

Although mTOR inhibitors have been used in clinical routine for more than 20 years, the kidney specific renal function of the mTORC1 and mTORC2 complexes remained unclear. Using kinase complex specific conditional knock-out animals for Raptor and Rictor, essential scaffolding proteins for mTORC1 and mTORC2 respectively, we could demonstrate a variety of renal specific functions for both kinase complexes. Furthermore, we focus on the cross-talk of mTOR signalling pathway, autophagy, and metabolism using comprehensive proteomics and transcriptomics approaches.

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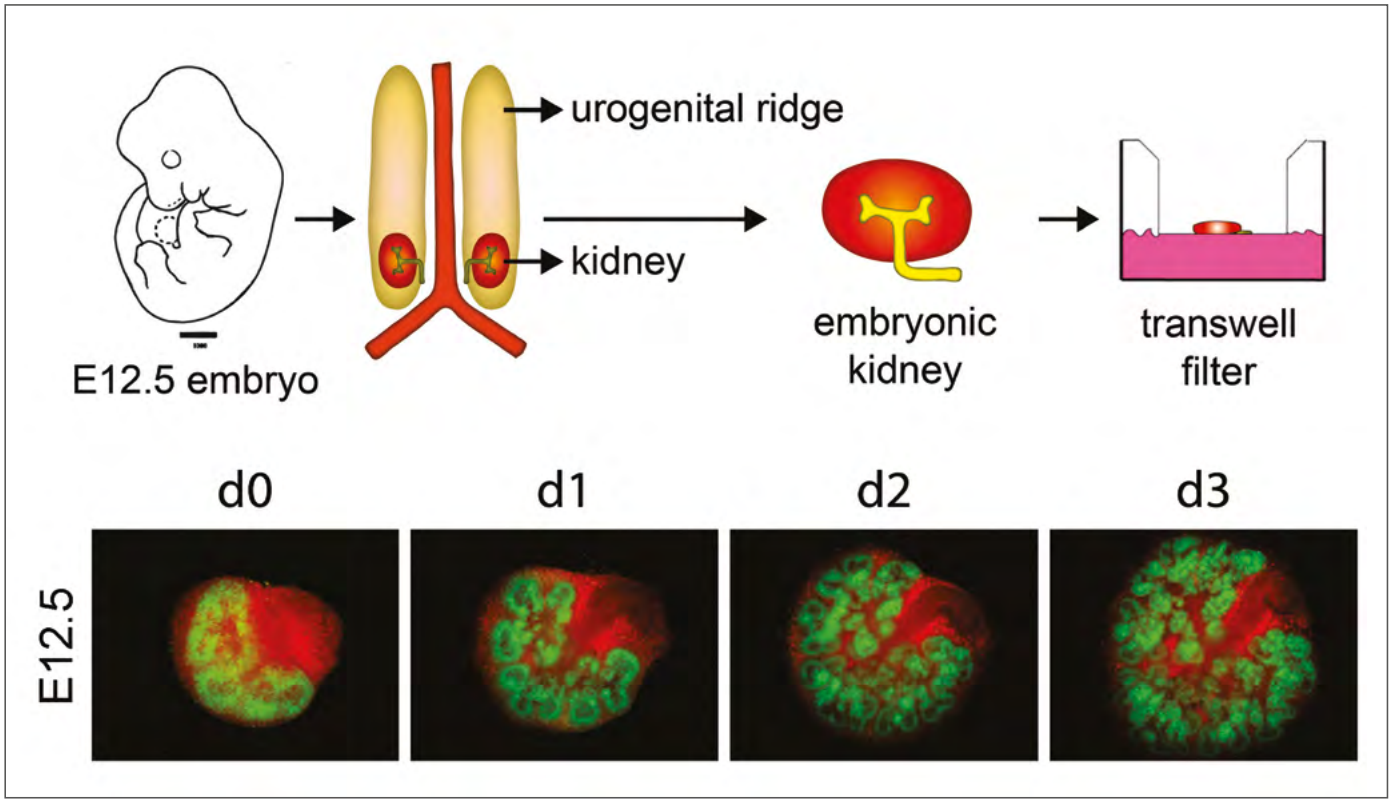

Isolation and growth of metanephric organs. Metanephric kidneys are isolated on embryonic day (E)12.5 and grown on a filter. Ureteric bud branching and nephron development continues in vitro over several days. Picture: Nicola Wanner

Drosophila model system to study kidney diseases

Nephrocytes, the "storage kidneys" of Drosophila, are highly reminiscent of mammalian podocytes. Transmission electron microscopy reveals the presence of a basal membrane and a slit diaphragm known from the mammalian filtration barrier. We utilize the nephrocytes as a platform to identify genes contributing to the slit diaphragm by an RNAi mediated suppressor screen and as a highly accessible model to study nephropathies.

METHODS

A. High purity cell sorting of glomerular cells

A.1 Fluorescence-based renal cell sort

The kidney is composed of a large number of specialized cell types that form a complex architectural dynamic. The glomerulus with several specialized cell types forms the filtration unit in the kidney and is the weak link in many acute and chronic kidney diseases.

To isolate cells from this compartment, the kidneys are perfused *ex vivo* via the renal artery with paramagnetic beads. Due to the size of the beads, they get enriched in the glomeruli, where the capillary diameter is the smallest. After several mechanic and enzymatic disruption processes and sieving steps, the glomeruli can be separated from the tubular and stromal cell types of the kidney via a magnet. Subsequent enzymatic disruption of the cells leads to a single cell dispension which can be used for fluorescence activated cell sorting of previously labelled cell compartments.

Using this protocol, glomerular cells can be highly enriched before the high purity isolation of the labelled cell type.

A.2 Antibody-based renal cell sort

Following the above described protocol, glomerular cell types can also be isolated using intracellular or extracellular staining protocol. This method has been established for pulse-chase experiments of inducibly labelled cell types after an aging period or kidney injury model to test for cell regeneration.

B. Metanephric organ culture as a screening tool for pharmacological agents

Metanephric organoids can be grown *in vitro* on a filter and continue with ureteric bud branching and nephron development. Using this organ culture setup, the culture medium can be supplemented with pharmacological compounds. The developing organs can be screened for growth impairment, cap mesenchyme population, ureteric bud branching, and nephron development (tubular compartments, glomerular cells). The cultures can also be used for RNA (qPCR, RNA-Seq) and DNA analyses.

C. Humanized mouse model for glomerular diseases

Phospholipase A2 receptor (PLA2R) and thrombospondin type-1 domain-containing7A (THSD7A) are commonly targeted podocyte autoantigens in idiopathic membranous nephropathy. Translating this knowledge into a lucid pathomechanistic understanding of involved immune-complex formation and subsequent molecular injury signalling, however, has been hampered by the absence of PLA2R in rodent podocytes. Using RNA-sequencing analysis of freshly isolated, pure murine podocyte fractions, we found that, unlike PLA2R, THSD7A is highly and specifically enriched in podocytes. In addition, injection of THSD7A abs or THSD7A positive MGN patient serum induces proteinuric disease in mice. Our findings indicate that there is a unique opportunity to rapidly transfer the discovery of THSD7A into experimental models, which should facilitate understanding of the molecular pathophysiological events that drive human idiopathic membranous nephropathy.

D. Model organisms for renal research

Our group devised methods for introducing mammalian kidney genes into filtering-like drosophila cells (nephrocytes). This system can be used to efficiently study the potential function of renal molecules in an easy to manipulate genetic system.

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Biochemistry/Structural Biology

HEAD Prof. Dr. Carola Hunte

PROJECTS

Architecture and Mechanism of Membrane Signalling Complexes

Cellular signalling is mediated through protein assemblies of different complexity that operate as integrated circuits. The group pursues a structural biology approach to provide insights in architecture and organization principles of membrane signalling complexes for a better understanding of the mechanisms of signal transduction at and across membranes. This knowledge will pave the way to challenge and control signalling processes by synthetic approaches.

Respiratory complexes and related supercomplexes

Reactive oxygen species (ROS) act as signalling molecules in the maintenance of physiological cell function and can also cause oxidative damage. Respiratory complexes I and III are the main ROS producers in oxidative phosphorylation. The control of ROS generation in mitochondria is not understood and the molecular links from the ROS source towards redox-signalling are not known. We determined the X-ray structure of the catalytic core of mitochondrial complex I, which is one of the largest membrane protein complexes known (Zickermann, Wirth *et al.* 2015). The complex generates about 40 percent of the driving force for mitochondrial ATP synthesis and its dysfunction is implicated in myocardial infarction, neuromuscular diseases, and neurodegenerative conditions. The structure offered important clues on the catalytic mechanism of complex I in line with a conformational switch that shuts down the mitochondrial enzyme under conditions that would promote excessive ROS formation. In complex III, ROS production appears to be modulated by the energetic state of the mitochondrial membrane. We showed that the signature motif of the catalytic quinol oxidation site is specific for phylogenetic lineages, named it Q_o motif and hypothesized that Q_o motif, redox potentials of electron transfer chains and quinone species co-evolved to control ROS generation (Kao and Hunte 2015).

Ion transport proteins

Integration in signalling networks controls the activity of ion transport proteins which are essential for homeostasis of ions, intracellular pH, and cell volume. The integration modulates transporter function in a cell-type specific manner and coordinates responses during metabolic and developmental changes. We structurally and functionally characterize selected ion transport proteins and their interaction with signalling partners. The studies will further the understanding of the mechanisms of transport and its modulation through signalling interactions.

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METHODS

Production of recombinant membrane proteins

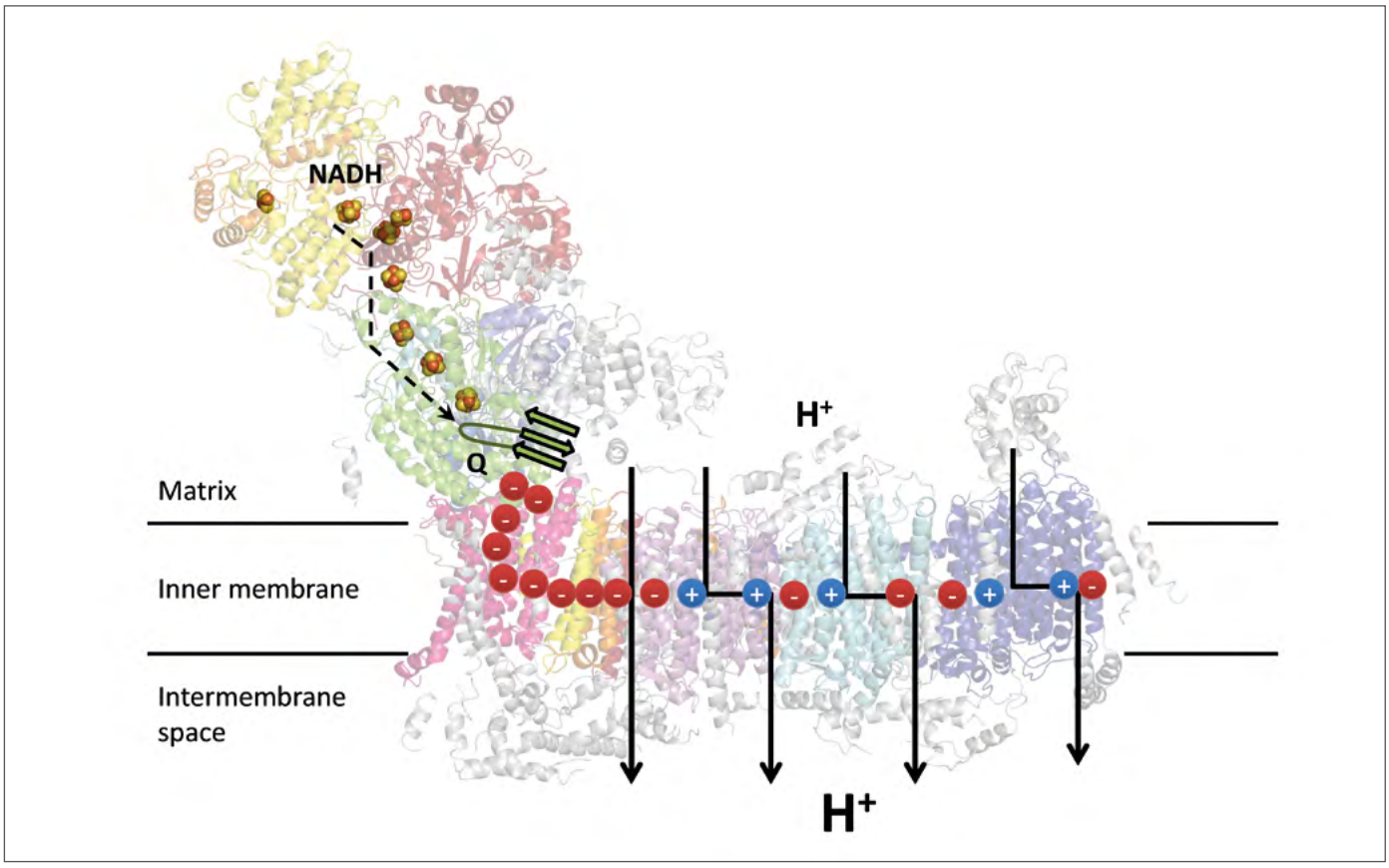
We use bacterial (*Escherichia coli, Corynebacterium-glutamicum, Paracoccus denitrificans*) and yeast (*Saccharomyces cerevisiae*, *Pichia pastoris)* expression systems for large scale production of prokaryotic and eukaryotic membrane proteins. Systematic analytical expression screens with diverse strains and tunable expression are established. Cell-free expression at large scale is available based on custom-made *E. coli* lysates. For yeast cells, glass bead mills show good performance for cell disruption and membrane protein recovery from analytical to large scale.

Detergents

The key to molecular studies of membrane proteins and their complexes is the detergent. We hold a comprehensive collection of detergents and other amphiphilic molecules. Systematic analytical screening procedures are available to identify the best compound for solubilisation, analysis, and purification. These include microscale fluorescent thermal stability assays (CPM assay) for purified proteins and analytical fluorescence-detection size exclusion chromatography (FSEC) protocols for GFP-fusion proteins in solubilized membranes.

Biochemical and biophysical characterization of purified membrane proteins

For purification of native and affinity-tagged membrane proteins, a broad scope of chromatography methods is established. An online fluorescence-detector is used to monitor GFP-fusion proteins. SEC is used for quality control and as final purification step prior to crystallization. In combination with multi-angle laser light scattering (SEC-MALLS), a precise determination of the oligomeric state of detergent solubilized membrane proteins is achieved. For soluble proteins, low volume state-of-the-art dynamic light scattering is used for fast analysis of monodispersity and thermostability as well as for buffer or ligand screening. Quantification and enzyme activity assays of cofactor containing respiratory complexes as well as ligand binding studies are facilitated by UV-VIS spectroscopy with minimal stray light and high dynamic range (Birth *et al.* 2014). Membrane protein reconstitution protocols for electrochemically tight proteoliposomes are established with defined protein-to-lipid ratio, lipid composition, and orientation. They facilitate enzyme activity assays and determination of proton-to-electron stoichiometry for respiratory complexes and transport assays for ion transport proteins. *In vivo* function of sodium/proton antiporter is analysed by semi-automated growth complementation



Structure of mitochondrial complex I with schematic presentation of mechanistic implications for redox-driven proton translocation. Figure: Wirth, Hunte. (See Zickermann, Wirth *et al.* 2015.)

assays. Analysis of binding interactions between proteins and signalling partners is typically characterized with SEC, blue-native (BN)-PAGE, and microscale thermophoresis. Structure/function relationships are probed by generation of defined mutations and consequent functional and structural characterization. Site-directed mutagenesis of mitochondrial encoded cytochrome b is facilitated by biolistic bombardment. Special attention is given to the role of lipids for structural and functional integrity of membrane proteins, membrane protein complexes, and supercomplexes (Hunte and Richers 2008). We develop methods for qualitative, quantitative, and structural analysis of lipids bound to membrane proteins.

Generation of recombinant antibodies for structural and functional studies

Antibody fragment mediated crystallization of membrane proteins was initiated by Prof. Dr. Hartmut Michel (Max Planck Institute of Biophysics, Frankfurt/Germany) and had a major impact on the advancement of membrane protein structure determination. Recombinant antibodies in Fv and Fab format are not only useful for crystallization but are versatile tools for structural and functional studies. An update of methods which we use for immunization, generation of recombinant antibodies from monoclonal hybridoma cell lines, and production of antibody fragments by periplasmic expression in *E. coli* was recently published (Mir *et al.* 2015). Hybridoma generation and culture are established, but are now replaced by phage display methods which provide fast access to high-affinity native binders.

Crystallization and X-ray structure determination

For structure determination by X-ray crystallography, purified proteins are subjected to comprehensive screening for crystallization conditions. Robotic liquid handling systems permit coverage of large crystallization space with protein amounts of less than 1 mg. Initial screens with 100 µg of protein are feasible. Crystal detection is supported by automated imaging systems with visible and UV light which take microscope images and document the crystallization set-ups over time. The automation permits a continuous crystallization pipeline beneficial for collaborations. Variation of detergent composition and lipid environment are critical parameters to establish crystallization conditions. An automated pipetting station supports preparation of lipidic cubic phase crystallization set-ups. Protein-antibody complexes or signalling complexes are subjected to *de novo* screening as crystallization conditions often vary compared to the protein alone. Antibody-mediated crystallization is especially attractive to stabilize thermolabile proteins and to trap defined conformations. Methods are established to crystallize low affinity complexes, large multi-subunit membrane protein complexes and membrane proteins with low affinity hydrophobic ligands. X-ray diffraction data of crystals are collected at synchrotrons. Customized software protocols are used for fast analysis of ligand binding. Methods for data collection of radiation sensitive protein crystals, phasing for structures of large complexes at low resolution, and model building at medium resolution were extensively optimized (Hunte *et al.* 2010; Zickermann, Wirth *et al.* 2015).

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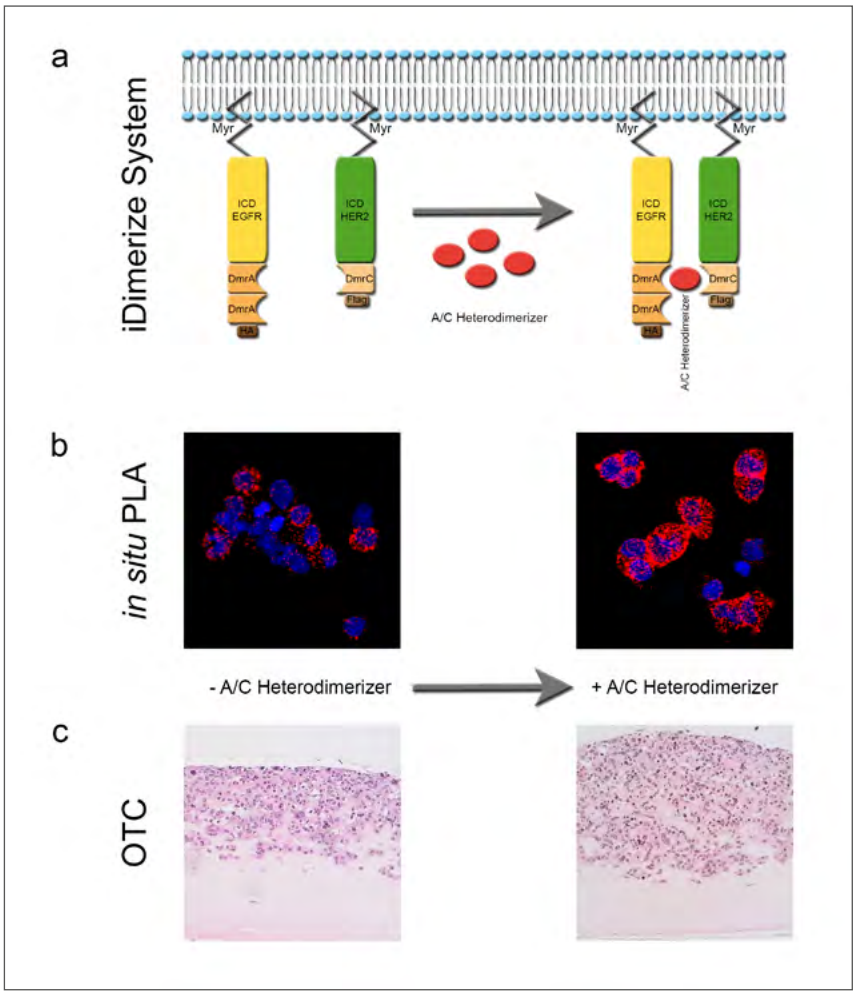
Molecular Tumorpathology

HEAD Prof. Dr. Silke Laßmann

PROJECTS

Our research group is interested in carcinogenesis and therapy-prediction of solid tumours, with a focus on the identification of mechanisms of action and resistance of gastrointestinal tumours to ErbB receptor family inhibitors and to drugs targeting epigenetic modifiers.

Moreover, we further develop molecular pathology analyses in fixed tissue specimens for translational research and for molecular pathology diagnostic applications.



The figure shows the scheme of constructs used for controlled dimerization of e.g. EGFR/HER2 heterodimers by A/C heterodimerizer (a), the associated validation of EGFR/HER2 dimerization in transduced esophageal cells by proximity ligation assay before and after A/C heterodimerizer application (b), and the morphological analysis (H&E stains) of transfected cells in organotypic cultures without and with A/C heterodimerizer. Source: Dr. Christiane Fichter

bioss.science/pm/molecular_tumorpathology

Selected project with focus on oncogenic signalling: ErbB receptors in esophageal cancer

Our group works on ErbB family dimerization and signalling involved in early development (initial invasion) and progression (metastasis) of esophageal cancer. We particularly study the effect of ErbB signalling on esophageal epithelial cell organization and initial invasion. Thereby the influence on mesenchymal and amoeboid type cell migration and invasion is also analysed more closely. With this, we try to gain detailed knowledge about the earliest steps of ErbB driven esophageal epithelial cell carcinogenesis and associated risk factors, the importance of ErbB driven pathways for esophageal cancer cell motility and associated metastatic predictors as well as the preclinical insight into the feasibility of using ErbB family inhibitors in ESCCs and BACs.

METHODS

***In situ* proximity ligation assay (*in situ* PLA)**

In situ PLA (Duolink) allows detection and quantification of homo- or heterotypic protein interactions in cell or FFPE tissue samples. Depending on whether you want to detect homo- or heterotypic protein interactions, primary antibodies directly conjugated with oligonucleotides or oligonucleotide labelled secondary antibodies are used. If the proteins of interest interact with each other and the oligonucleotide labelled antibodies are in close proximity (<40 nm), added oligonucleotides hybridize, are ligated, and then amplified via rolling circle amplification. Fluorescently or horseradish peroxidase labelled oligonucleotides allow detection of the interaction with fluorescence or brightfield microscopy.

iDimerize System

The iDimerize technology (Clontech) allows controlled induction of interactions between two proteins. To create such an inducible system for protein-protein interactions, the protein(s) of interest are tagged with dimerization domains Dmr A or C. Small molecule dimerizer ligands are designed to bind two dimerization domains. Hence, addition of these dimerizer ligands induces interaction of the proteins fused to the dimerization domains. Homotypic or heterotypic interactions can be induced by addition of A/A Homodimerizer or A/C Heterodimerizer, respectively.

Three dimensional organotypic culture (OTC)

In three dimensional OTCs an extracellular matrix mainly consisting of Matrigel and collagen is organized by fibroblasts for some days. Then esophageal epithelial cells or esophageal cancer cells are plated on the surface of this matrix. The epithelial cells grow in this culture for one to two weeks. Exposure of the epithelium to air and creation of a liquid-air interface promotes epithelial stratification and differentiation. Finally, the OTCs are harvested for histology or other applications such as RNA/protein analyses. This model system is a kind of tissue engineering and allows analysis on cell differentiation, cell invasion, or epithelial-stromal interactions.

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Developmental Biology and Biotechnology of Plants

HEAD Prof. Dr. Thomas Laux

PROJECTS

The spatial organization of stem cell niches is stably maintained in plant meristems despite a changing cellular context, but the underlying mechanisms for this are largely unknown. In our group we investigate how the spatial organization of the *Arabidopsis* shoot and root meristems are maintained. The shoot meristem stem cell niche works in a population mode: stem cell divisions are random and the fate of stem cell daughter cells entirely depends on positional signalling rather than asymmetric inheritance (Aichinger *et al.* 2012). Because all cells divide, including the niche cells, cell fate regulation in the shoot meristem provides a paradigm to study cell-cell communication of a self-organizing stem cell system. A signalling feedback loop between the stem cells and the subtending organizing centre, mediated by WUSCHEL (WUS) and CLAVATA3 (CLV3) activities, regulates the balance between stem cell maintenance and differentiation. We have recently shown that two opposing signal pathways determine the region of stem cell competence by regulating responsiveness of cells to the WUS/CLV3 signals (Knauer *et al.* 2013; Tucker *et al.* 2008).

One of these signals is the *miR394*, which acts as a mobile signal produced by the surface cell layer (the protoderm) conferring stem cell competence to the distal meristem by repressing the F box protein LEAF CURLING RESPONSIVENESS (LCR). This repression is required to potentiate signalling from underneath the stem cells by the transcription factor WUSCHEL, maintaining stem cell pluripotency. The interaction of two opposing signalling centres provides a mechanistic framework of how stem cells are localized at the tip of the meristem. Although the constituent cells change, the surface layer provides a stable point of reference in the self-organizing meristem.

Currently, we are investigating the *molecular functions of LCR*. As an F-box protein, it is likely involved in the targeted degradation of proteins, although also other functions for F-box proteins have been reported. To find LCR interacting partners, and potential degradation targets, we employ protein protein interaction screens, genetic screens, and candidate approaches.

bioss.science/pm/developmental_biology_biotechnology_plants

METHODS

***In situ* techniques**
Detection of mRNA and miRNA of tissue sections and whole mount samples, including the use of LNA probes.

miRNA sensors
Expression of miRNA-sensitive and miRNA-resistant reporter genes allow for the localization of miRNA activity.

INTACT (isolation of nuclei tagged in specific cell types)
Here two proteins are expressed in a tissue specific manner, nuclear targeting fusion protein (NTF) and BirA. NTF is located to the nuclear envelope and is biotinylated by BirA where the expression of the two proteins overlaps. Thus, it is possible to extract very specific nuclei with the help of Streptavidin beads.

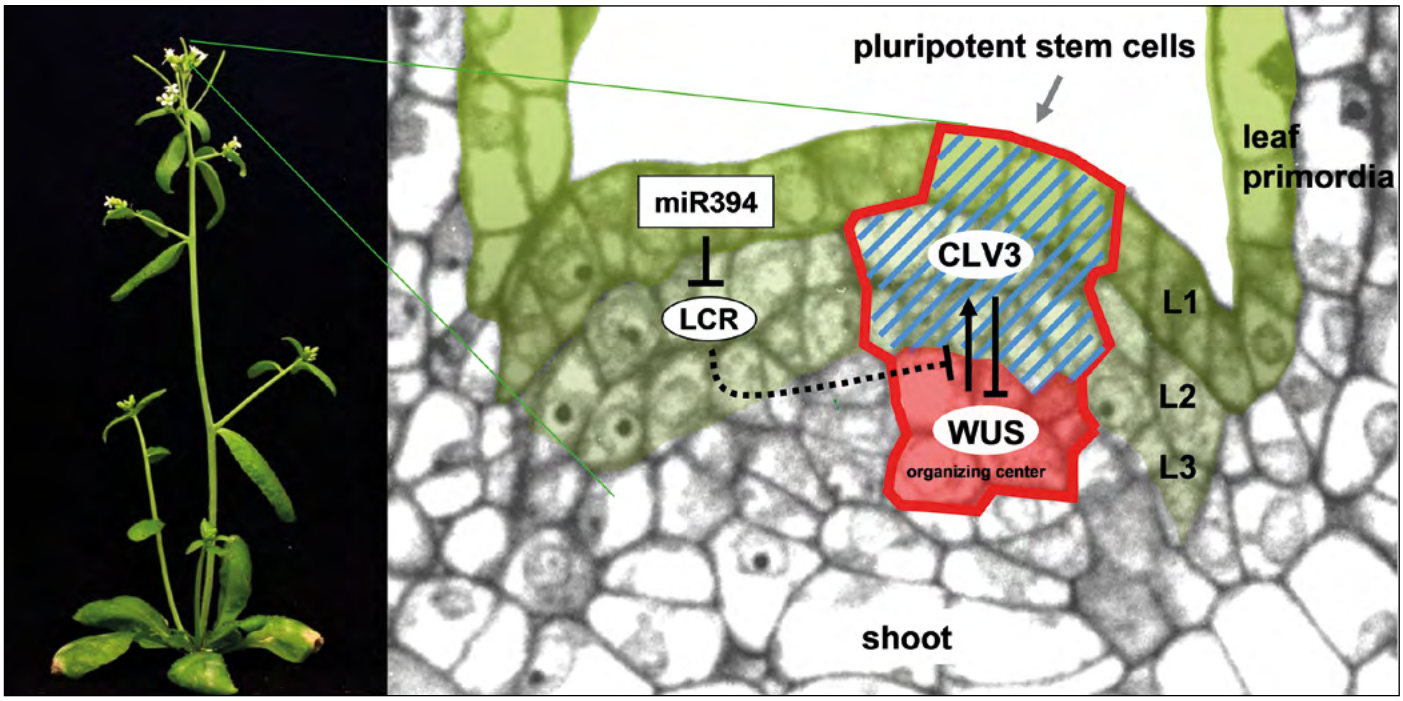
Microscopy
is a very important tool for our lab for example for *life imaging* of *Arabidopsis* root tips.

EMSA, Yeast-one-hybrid, ChromatinImmuno-Precipiation, ChIP-on-ChIP are routinely performed in our lab.

Protein-protein interaction screens
Beside Yeast-2-Hybrid studies *(Y2H)* we perform Immunoprecipitation *(IP)* from GFP tagged proteins expressed in a tissue specific, inducible manner with subsequent identification of interacting proteins by mass-spectrometry. Furthermore, we are establishing screens based on bifluorescence complementation *(BiFC)* in protoplasts and *E. coli.*

BiFC
We are using this technique for verification of interacting partners. As BiFC is highly sensitive towards false positives when interacting partners are overexpressed, we are establishing a triple BiFC approach using split CFP, split YFP, and split mCherry in a transient expression system *(Nicotiana Benthamiana)*. This allows for simultaneous positive control (transformation), negative control, and testing of one interaction. Furthermore, the compatibility between split CFP and split YFP is used to investigate competitive binding between interacting partners.

CRISP/Cas9 technique
We are using this technique for creating knock-out lines of genes for which classical T-DNA insertion lines are not available.



We investigate the regulation of stem cells in the shoot meristem (right) of the model plant *Arabidopsis thaliana* (left). Stem cell competence is restricted to the three outermost cell layers of the shoot meristem, L1-L3 by, a gradient of miR394, that is exclusively produced in the outer epidermis. By degrading the mRNA of the stem cell inhibitor LCR, the miRNA394 allows cells to repond to the mobile transcription factor WUS, which switches on the pluripotency programme. All other cells will undergo differentiation and form the shoot with leaves, flowers, and branches.

Greengate (Goldengate) cloning

In our lab we have experience with several different cloning strategies (classical, Ligation independent cloning, gateway, TOPO). Lately we introduced the Greengate cloning system, which is an adaptation of the Goldengate standard for plants.

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Microfluidics and Bioengineering (MiBioEng)

HEAD Dr. Matthias Meier

PROJECTS

***In situ* Sequencing to reveal Cell Signalling Dynamics**

We integrate immuno-PCR technology based on the proximity ligation assay (PLA) on microfluidic large-scale integration (mLSI) chip platforms to study signalling networks in the context of cell development. PLA is an established biochemical benchtop assay, which enables the visualization and quantification of single proteins, protein modifications, and protein interactions. With hundreds of autonomous cell cultures on mLSI chips, we study the Akt signalling pathways *in situ* under rapidly changing cellular microenvironments resembling conditions in the human body (Blazek *et al.* 2014). A central technological aim of this project is to multiplex protein detection by applying sequencing by ligation technology on DNA-labelled antibodies. The sequencing approach will allow us to correlate protein events and their statistical variations in single cells during signal transduction.

Artificial Micro-Organs on Chip for 3D Signal Transduction

One of the most prevailing questions within the field of cell signalling is: Do the results obtained from model cell lines in artificial environments resemble the dynamics of cell signalling in multicellular tissues or organisms? Cells embedded in three-dimensional (3D) tissues have been shown to receive mechanical signals from cell-to-cell contacts and the extracellular matrix. The mechanical properties of the cell microenvironment can alter cell size, growth, proliferation, and gene expression. In this project, we are developing microfluidic devices to culture 3D cell spheroids formed with human adult stem cells. On chip, we control the microenvironment and differentiate the stem cells over weeks. With the help of high-throughput imaging methods, we aim to reveal signal expansion between and in cells embedded in the tissue-like structures.

Biosensors on a microfluidic chip

Metabolites are central signalling transmitters in the regulation of cellular homeostasis. Alteration of a vast variety of metabolites has been observed in diseases such as cancer and obesity. In many cases, however, it is not clear if and how metabolites transmit signals to regulate or dysregulate cellular functions and growth. To answer this question, we engineer biosensors based on protein binding domains and natural RNA aptamers for real-time fluorescence monitoring of sugars and amino acids (Ketterer *et al.* 2014). For direct engineering of proteins and RNAs, we use mLSI technology combining *in vitro* protein or RNA synthesis with a miniaturized affinity assay (mIP). Generation and thermodynamic characterization of large protein and RNA mutation libraries can thus be streamlined by iterative selection and mutation rounds to elucidate the thermodynamics of binding and stability of these molecules.

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METHODS

Protein, RNA, DNA Interaction Measurements on Chip

With help of the mLSI technolgy chip we are able to screen rapidly interactions between various biomolecules (Meier *et al.* 2013). The chip technology combines on-chip *in vitro* protein or RNA synthesis with a miniaturized immuno-affinity assay (mIP). On the chip co-spotted DNA microarray containing linear template encoding the bait and prey pairs is bonded to the microfluidic device. *In vitro* expression of the proteins on the chip is achieved by using *in vitro* transcription/translation systems. With this hybrid technique, it is possible to express thousands of protein combinations on chip without the need for purification. Interactions are then tested with the mIP, where baits and prey are expressed with suitable tags. Further, the chip integrates a previously developed mechanical trapping of molecular interactions, which allows one to measure interactions without losing proteins due to washing and can thus detect weak or transient interactions.

Photo- and Soft Lithography

Photolithography and multilayer soft lithography are used to fabricate microfluidic chips with various polymers. Initially, photolithography is used to produce masters with feature sizes ranging from one to hundreds of micrometers. The masters can then be used to rapidly prototype microfluidic chips, e.g. with materials as for example polydimethylsiloxane (PDMS), polymethylacrylat (PMMA), or polyurethane (PU). The shared clean room facilities at IMTEK offer a vast variety of machines and protocols, for example plasma-, lamination-, and adhesion layer bonding or etching glass and silicon substrates. My lab is specialized in the design and production of multilayer PMDS devices integrating membrane valves to build complex flow logics, which are regarded to be analogous to electronic integrated circuits (Unger *et al.* 2000, Meier *et al.* 2012).

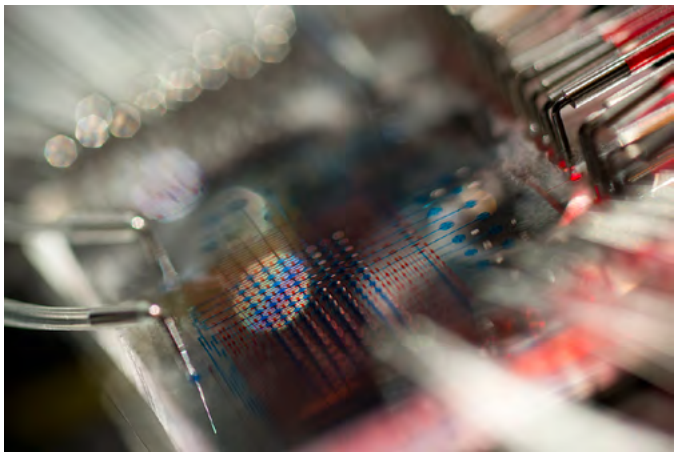
Automated Cell Culturing

Cell culturing can be integrated on microfluidic chips to streamline automated and highly precise cell manipulation and analytical workflows. Moreover, microfluidic cell cultures offer dynamic control of the cell microenvironment with high spatial and temporal resolution, down to one microns and a millisecond, respectively. We have chip platforms holding up to 512 autonomous cell cultures for real time or immuno-fluorescence imaging (Blazek *et al.* 2013). Our cell culture chips are compatible with adhered or non-adherent cell types, where cells can be cultured from one day to two weeks in order to study for example differentiation processes. The main advantage of the on chip cell culture process is a significant reduction of material consumption and handling time.

High-throughput cell imaging and analysis. For the cell culture chip platforms, we always have developed in parallel a library of image analysis functions and programmes to streamline high-throughput image acquisition and analysis. Tools are written in Matlab (Mathworks) programming environment and can be accessed within project cooperation.

Metabolic Click labelling

Click chemistry is a simple two-step chemical reaction used to label proteins temporally. Most often, the chemoselective click reaction between an azide and an alkyne is used for this purpose. We established specific azide- or alkyne-DNA labelling protocols for antibodies. Further, protocols and chip technologies have been established to implement metabolic labelling of cell cultures with amino acid analogs (AAA). For this, AAA are administered to the cell medium. Once the amino acid analog is metabolized, the click label is actively incorporated into all proteins within a cell. Similar to radioactive or isotope labels, the amino acid analog can be exploited in pulse-chase experiments. During the pulse phase, cells are exposed to the Click labelled amino acid analog, whereas natural amino acids are fed to the cells in the following chase phase. Further, we adopted and developed a technique to label proteins with ortholog tRNA/RNA synthetase from acheaebacteria. Baculovirus and Crisper/Cas vectors for genome engineering are available upon request or at the BIOSS Toolbox.



The figure shows the scheme of constructs used for controlled dimerization of e.g. EGFR/HER2 heterodimers by A/C heterodimerizer (a), the associated validation of EGFR/HER2 dimerization in transduced esophageal cells by proximity ligation assay before and after A/C heterodimerizer application (b), and the morphological analysis (H&E stains) of transfected cells in organotypic cultures without and with A/C heterodimerizer. Source: Dr. Christiane Fichter

3D Tissue cultures

Spheroidal clusters of cells are mimicking physiological tissues, while retaining the advantages of *in vitro* cell culture in terms of simplicity and reproducibility. Within a spheroid, a few hundred cells form strong cell-cell contacts and synthesize extracellular matrix and thus spheroids resembling the mechanical and communicational microenvironment of a tissue. We have established protocols to reproducibly produce spheroids by hanging drop approaches from different human cell types. Further, we are able to culture, stimulate, and process spheroids for immuno-staining on a mLSI chip in parallel fashion.

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Mitochondrial Signalling

HEAD Prof. Dr. Chris Meisinger

PROJECTS

Regulation of the mitochondrial protein import machinery by reversible phosphorylation

The preprotein translocase of the outer mitochondrial membrane (TOM-complex) provides the organelle's entry gate for most preproteins from the cytosol and has been considered to be constitutively active for long time. We found more than 30 phosphorylation sites on all subunits of the TOM-complex of yeast and identified several signalling mechanisms that adjust mitochondrial protein import rates to cellular demands. This includes cytosolic and mitochondrial protein kinases like casein kinase 1 and 2, Protein kinase A, and the cyclin-dependent kinase Cdk1 which regulate signal switches at the TOM-complex e.g. upon metabolic changes or during different cell cycle phases (Schmidt *et al.* 2011; Gerbeth *et al.* 2013; Harbauer *et al.* 2014; Opalinska and Meisinger 2015).

Rebuilding mitochondrial protein import mechanisms in artificial liposomes

Several mitochondrial outer membrane proteins appear to be imported independently of the protein import channel. We have determined the lipidome of the yeast outer membrane and generated liposomes that mimic the lipid composition of this membrane. We have screened a collection of preproteins for their import and assembly efficiency in liposomes lacking single lipid components and found a specific role of the signalling lipid phosphatidic acid in import of components of the mitochondrial fusion machinery. These findings provide an explanation of the largely unknown mechanism behind the role of phosphatidic acid as a fusogenic lipid.

Coupling of mitochondrial signal sequence processing and turnover

About 70 percent of mitochondrial preproteins contain an N-terminal signal sequence, which is required for organellar targeting and import (Vögtle *et al.* 2009). The majority of these signal sequences is processed upon import by the presequence protease MPP in the matrix. Cleaved presequence peptides are then further degraded by the peptidasome Cym1/hPreP. Presequence processing and degradation of presequence peptides were so far considered as independent processes. We found that impaired peptide turnover in the matrix inhibits presequence processing activity of MPP indicating a functional coupling of both processes. Moreover, Amyloid beta peptides, which were found in mitochondria of Alzheimer's disease patients, inhibit the peptidasome in the matrix thereby leading to impaired presequence processing activity. This mechanism led to a model how Amyloid beta peptides induce several mitochondrial dysfunctions in Alzheimer's disease by inducing a proteotoxic stress response (Vögtle and Meisinger 2012; Mossmann *et al.* 2014).

bioss.science/pm/mitochondrial_signalling

METHODS

Analysis of protein phosphorylation by Phos-tagTM SDS-PAGE

The phosphate affinity (Phos-tag™) SDS-PAGE method is employed to analyse phosphorylated proteins and has proven very useful for mapping the phosphorylation status of proteins, as it does not require radioisotope labelling or phospho-specific antibodies. We have employed these techniques for the analysis of phosphorylated mitochondrial and cytosolic proteins from the yeast *S. cerevisiae* (Schmidt *et al.* 2011; Gerbeth *et al.* 2013).

The Phos-tag™-manganese complex specifically interacts with phosphorylated proteins (phosphorylated forms of Ser, Thr and Tyr are equally recognized) and interferes with their mobility during electrophoresis, separating phosphorylated from non-phosphorylated protein species and even allows high-resolution separation of multiple phosphorylation sites within the same protein. The compound is added to the gel prior to polymerization and can be applied to standard SDS-PAGE as well as to Blue Native PAGE to study oligomeric protein complexes. Sample preparation as well as electrophoresis is performed according to standard lab protocols. Before transfer of the gel to PVDF membranes, the Phos-tag™ reagent is quenched by EDTA and Western Blotting is carried out using the wet transfer technique.

In vitro and *in organello* phosphorylation of mitochondrial proteins

To identify or confirm predicted mitochondrial phosphoproteins as well as to identify the putative protein kinases, we perform *in vitro* and *in organello* assays using isolated intact mitochondria or recombinantly expressed, purified mitochondrial proteins. The protein kinase screens are carried out using commercially available eukaryotic serine/threonine kinases. Alternatively, instead of recombinant mammalian kinases, yeast kinases were affinity-purified under native conditions. Furthermore, freshly prepared yeast cytosolic extracts can be applied for kinase screens and are especially helpful if defined cellular conditions, such as certain metabolic states or cell cycle stages, are analysed. Protein kinase screens are carried out by incubation of mitochondrial proteins with purified enzymes or cell extracts in a kinase-specific reaction mix containing essential cofactors supplemented with ATP (or radiolabelled ATP). The assay might also be supplemented with kinase inhibitors or activators. Furthermore, protein dephosphorylation with alkaline phosphatase is also a helpful tool to ensure that a protein is indeed phosphorylated. Depending on the experimental composition, detection and analysis of the phosphorylation assay is performed using Phos-tag™, autoradiography, or immunodecoration with a phosphospecific antibody.

Protein import and assembly in liposomes

We have established a procedure that allows monitoring of protein import and assembly into synthetic liposomes that mimic the lipid composition of the mitochondrial outer membrane. This allows testing of import efficiencies in the presence or absence of a distinct lipid to find a direct requirement for the respective lipid species. The procedure requires the generation of radiolabelled preproteins by *in vitro* transcription/translation in the presence of [35S]methionine. Radiolabelled preproteins are then incubated with liposomes followed by a sedimentation ultracentrifugation step, which removes non-imported preproteins. A sucrose gradient flotation ultracentrifugation step will then separate intact liposomes from aggregated or non-soluble material and reduce the background binding of radiolabelled precursors. Liposomes are loaded on SDS-PAGE and imported preproteins are visualized by autoradiography. Alternatively, samples may be lysed in mild non-ionic detergents and separated via blue native PAGE. This allows monitoring of a potential assembly step of the preprotein in an oligomeric form. To analyse membrane insertion, carbonate extraction (100 mM Na_2CO_3 (pH 11.5)) followed by a 100.000x g centrifugation step separating integral and peripheral membrane proteins can be applied. To test the correct topology of membrane-inserted preproteins, a mild protease treatment (e.g. Proteinase K or Trypsin) can be performed and proteolytic patterns (visualized by SDS-PAGE and autoradiography) are compared with mitochondrial-imported preproteins treated with the same protease after import reaction *in organello*.

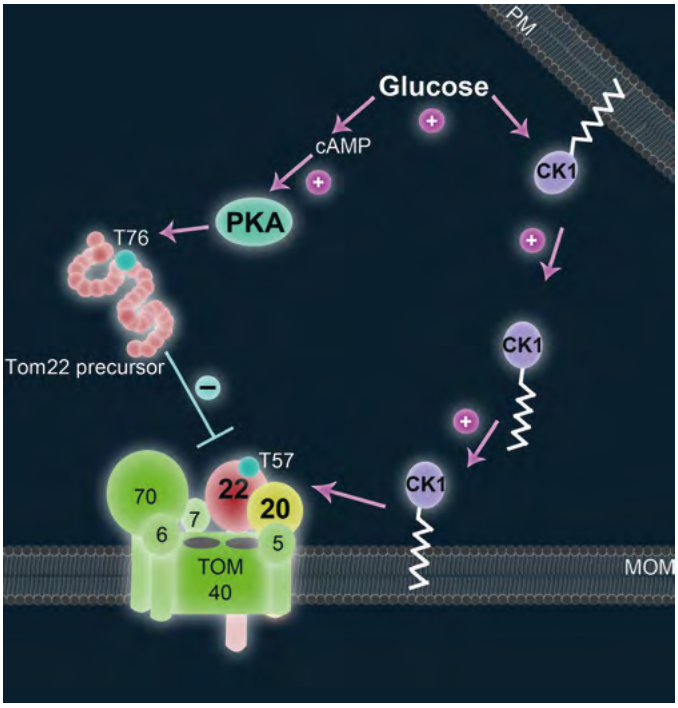
Measurement of mitochondrial protein and peptide turnover

Mitochondrial protein degradation can be analysed *in vivo* or *in organello*. For *in vivo* turnover cytosolic translation of mitochondrial preproteins is blocked by treatment of a yeast cell culture with cycloheximide. Cells are further grown and harvested at various time points. Factors influencing protein's half-lives and mitochondrial proteome turnover can be easily modulated, e.g. by changes in the growth temperature or the carbon sources. Analysis is performed by post-alkaline extraction of whole cell yeast extracts followed by SDS-PAGE and immunoblotting (Vögtle *et al.* 2009).

For analysis of mitochondrial protein turnover *in organello* isolated mitochondria are incubated in sterile isotonic buffer. At various time-points samples are taken, washed, and mitochondria analysed by SDS-PAGE and immunodecoration. *In organello* protein turn-over can be manipulated by addition

of protease inhibitors, e.g. complete cocktails or specific protease class inhibitors.

To study peptide and protein degradation independent of *in organello* mitochondrial protein import, soluble mitochondrial fractions are used. This allows the addition of synthetic peptides, chemical amounts of proteases or radiolabelled preproteins that would otherwise not be imported into the organelle. Soluble mitochondrial fractions are obtained by sonication of isolated yeast mitochondria followed by ultracentrifugation at 100 000 xg for 30 minutes at 4°C to separate membrane fractions from the supernatant which is used for peptide degradation assays (Mossmann *et al.* 2014). For analysis of mammalian mitochondria, isolated organelles are lysed with the mild detergent digitonin followed by centrifugation to obtain the soluble mitochondrial fraction. The soluble mitochondrial fractions can be obtained from various strains, e.g. protease mutants, and can be used for peptide degradation assays by addition of synthetic peptides and monitoring their degradation over time (Mossmann *et al.* 2014). Cell-free translated peptidases (produced in a wheat germ based expression system) can be added to manipulate peptide degradation activities in the assay. Samples are incubated at different temperatures for various time points and analysed on SDS-PAGE followed by immunodecoration.



Glucose induced regulation of the central mitochondrial import receptor Tom22 by Protein kinase A (PKA) and Casein kinase I (CK1).
Image: Magdalena Opalinska and Chris Meisinger (2014). Mitochondrial protein import under kinase surveillance.
Microbial Cell 1 (2): 51-57; DOI: 10.15698/mic2014.01.127

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Photograph by: Max Planck Institute of Immunobiology and Epigenetics

Proteomics Research Facility, MPI-IE

HEAD Dr. Gerhard Mittler

PROJECTS

Quantitative Proteomic Analysis of the interactome of MARs (nuclear Matrix Attachment Regions):

Our group has a long-standing interest in understanding gene regulation and nuclear organization at a systemic level. We have recently reported an in-depth proteomic characterization of a subnuclear structure termed the nuclear matrix[1]. In a joint effort with the Grosschedl department we are now in the process of cataloguing the trans-acting proteins, which are binding to composite enhancer-MAR elements and MARs. In order to achieve this we employ a reverse ChIP proteomics approach (methods).

MARs represent AT rich regulatory DNA sequences in metazoan and plant genomes and are believed to organize chromatin within the cell nucleus by mediating the binding of DNA to the nuclear matrix[1/2] thereby compartmentalizing chromosomes into functional domains. These regions appear to be functionally conserved, can be found close to boundaries of transcribed regions and are often flanking other regulatory elements, like enhancers. Interestingly, stable transgenes flanked by particular MARs exhibit copy-number and position-independent expression levels, which has found a wide potential usage for gene therapy vectors and heterologous protein production systems.

We are particularly interested in elucidating the protein-binding landscape of two MARs that both have an established biological function in mammalian cells. One of them is represented by two (~300 bp) matrix attachment region sequences flanking the 5´ and 3´part of the intragenic enhancer Eμ of the murine immunoglobulin μ heavy chain locus[3]. This enhancer-MAR element is important for robust B-cell-specific expression of the Ig μ heavy chain at the pro- to pre-B cell stage of B cell development. The second MAR of interest is located 5' to the human β interferon gene[4] and constitutes the most frequently utilized MAR sequence in biotechnology. Towards this end, we find that different MAR sequences recruit shared common but also individual MAR element-specific proteins.

bioss.science/pm/proteomics_research_facility

METHODS

Nanoscale LC-MS

The unit is running two quadrupole orbitrap instruments (QExactive, QExactive Plus), one hybrid linear ion trap (LIT) FT-MS instrument (Orbitrap XL+ETD), and one LIT instrument (LTQ), that are coupled online via electrospray ionization (ESI) source interfaces to nanoLC systems. We use both *nanoHPLC* (Agilent, Thermofisher-Proxeon) as well as *nanoUHPLC* (Thermofisher-Proxeon, Thermofisher-Dionex) for upfront reversed-phase (RP) separation of complex peptide mixtures generated by proteolytic (mostly trypsin) digestion of proteomes. NanoUHPLC enables the use of columns with a length of up to 50 cm that in conjunction with sub-2µm bead capillary columns offer superior separation power (increased peak capacity, sharper peaks) that is best combined with very fast scanning mass spectrometers of the QExactive series.
Our equipment allows us to employ the most commonly used fragmentation technologies (*CID, HCD, ETD, MSA*) for MS/MS identification and PTM analysis of proteins, enabling the in depth characterization of proteins and protein complexes.

Sample preparation and offline prefractionation

Depending on the sample, tryptic digestions are performed either *in-gel*, in solution *(iST),* or by *FASP* (filter-aided sample preparation).

Sample preparation by SPE (solid phase extraction) is performed offline (semiautomated) in a microcolumn-in-a-tip *(STAGE tips)* format (robust, reproducible, minimizing contaminations). The microcolumn set-up is very flexible and can accommodate reversed phase (standard), strong anion and cation exchange *(SAX, SCX)*, *HILIC* (hydrophilic interaction chromatography) as well as affinity chromatography (e.g. *Titania-MOC* for phosphor peptides) beads. For determination of asparagine (asparagine) glycosylation sites, we have adopted *Glyco-FASP* and *Zic-HILIC* in combination with N-gycosidase F mediated [18O]-labelling of glycosylation sites.

Pre-fractionation of very complex samples is done either at the protein level by 1D SDS-PAGE or at the peptide level via peptide-SAX or IEF.

For offline protein and peptide chromatography GE Biosciences microbore HPLC systems (SMART and Ettan LC) are used. Isoelectric focusing (IEF) can be performed on an Agilent 3000 off-gel fractionator.

Quantitative Proteomics and data analysis

The unit is very experienced in metabolic labelling of primary and transformed cell lines by the *SILAC* methodology and is able to produce substantial amounts of SILAC-labelled cells in bioreactors (spinner culture). Relative quantification of SILAC-MS data is currently achieved with the help of the MaxQuant and Perseus software environment. For conducting label-free quantitative proteomics experiments, we make use of the MaxQuant LFQ algorithm. The in-house bioinformatics pipeline additionally consists of a PEAKSTM workstation connected to a MascotTM Server for automated database searching.

Reverse ChIP

Our group has developed an approach termed SILAC-DNA-protein interaction screening[5] that is complementary to the classical chromatin immunoprecipitation (ChIP) method since our technology reveals proteins that are able to specifically interact with functional DNA elements of interest and does therefore represent a reverse ChIP methodology[6].

Briefly, designed DNA baits (length up to 3 kb) are immobilized on streptavidin magnetic beads using equimolar amounts of WT and control (mutant, scrambled, reverse sequence) DNA sequences thereby enabling reliable estimation of protein specific enrichment. Nuclear extracts are obtained from SILAC-labelled cell cultures and subsequently used for DNA pull-downs. After extensive washing, beads containing both samples are mixed and enriched proteins are eluted by nuclease (restriction endonuclease or benzonase™) digestion of the baits. Following tryptic cleavage proteins are subsequently subjected to LC-MS analysis.

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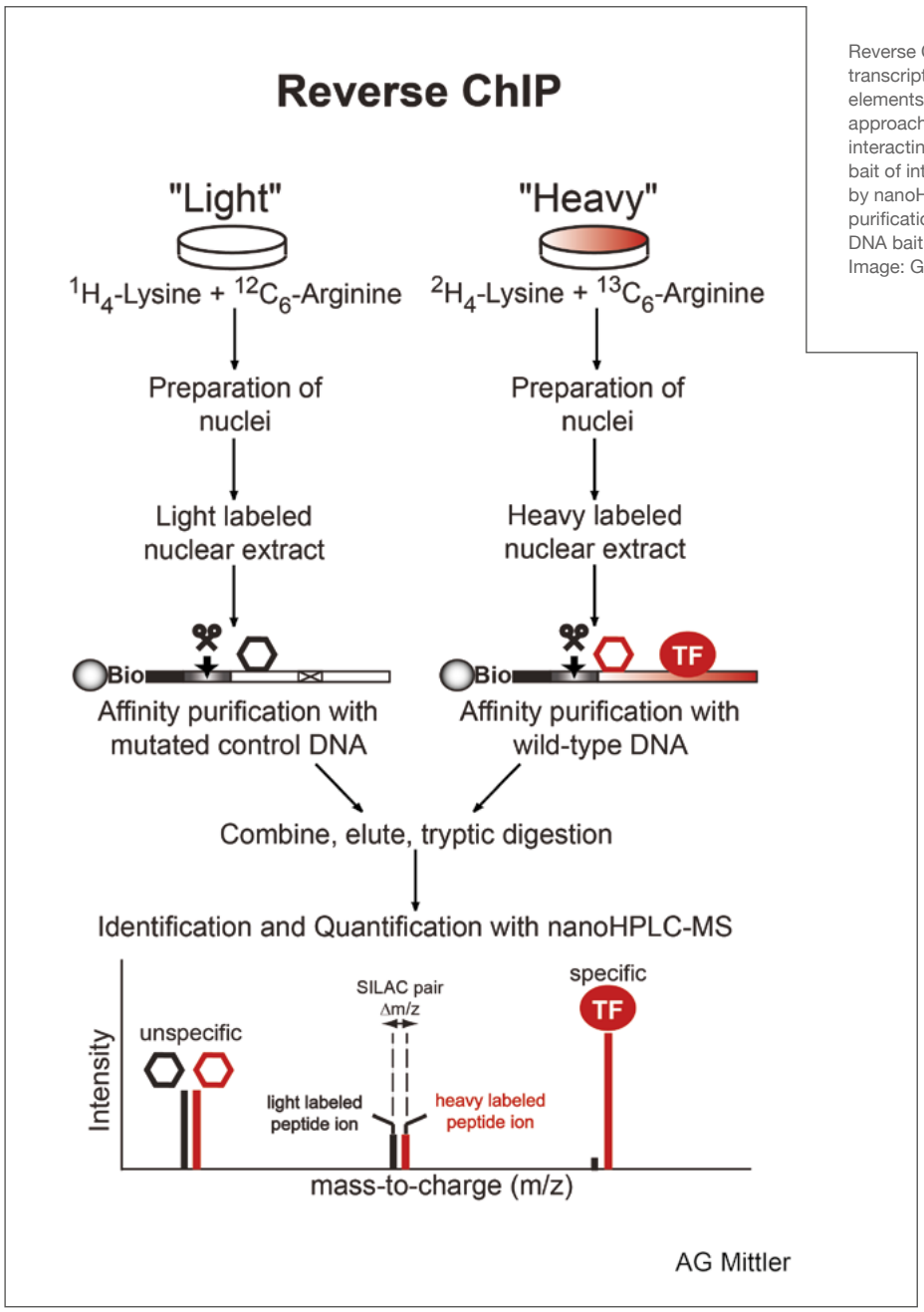

Reverse ChIP workflow for the identification of transcription factor (TF) binding to functional cisregulatory elements by a SILAC-based quantitative proteomics approach. TFs present in mammalian cell nuclear extracts interacting specifically with a functional DNA sequence bait of interest are monitored (as tryptic peptides) by nanoHPLC-MS subsequent to a one-step affinity purification pull-down. Non-functional (mutated) control DNA bait serves as a specificity control.
Image: G. Mittler

Gene Activation and Pluripotency in Development

HEAD PD Dr. Daria Onichtchouk

PROJECT

At the beginning of the embryonic life, the genome is not active and development depends only on maternally deposited products. Transition of the embryonic cells from quiescent to active state occurs during Zygotic Genome Activation (ZGA), which marks the start of the transition from maternal to zygotic control over the developing embryo. Currently, the exact mechanism of ZGA is not resolved. After ZGA the vertebrate embryonic cells are pluripotent – able to develop as any type of embryonic tissue. Starting from the beginning of gastrulation, pluripotency is gradually lost and cells become determined towards different embryonic lineages. The broad goal of our research is to understand the mechanism of ZGA and pluripotency in vertebrates, using zebrafish (Danio rerio) as a model system.

Epigenetics of ZGA

In zebrafish, conversion of transcriptionally silent early cells to a transcriptionally active pluripotent cell population occurs within less than an hour at ZGA, and coincides with establishment of major histone modifications. This conversion may be viewed as a "reprogramming" event, where pluripotency is acquired synchronously by all cells and occurs with 100 percent efficiency. Thus, zebrafish ZGA provides an ideal model system to study the molecular mechanisms of acquisition of pluripotency. We aim to understand the causal relationship between epigenetic chromatin state and binding of the "pluripotency transcription factors" Pou5f3, SoxB1, and Nanog to DNA. In zebrafish, Pou5f3, SoxB1, and Nanog mutants are viable throughout gastrulation, which allows us to monitor the changes in epigenetic modifications in single or combined mutant backgrounds at successive developmental stages. We use chromatin immunoprecipitation coupled with microarray (ChIp-on-chip) to detect selected chromatin marks, MNase digest to detect nucleosome positioning and bioinformatics analysis of obtained results together with transcriptome data. This systematic approach will allow us to connect chromatin state, binding of pluripotency transcription factors, and early transcription into a Gene Regulatory Network (GRN).

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METHODS

The TALEN-based gene editing technique

This technique allows to introduce deletion or insertion mutations to the genes of interest. To obtain *nanog* and sox19b mutants, we have designed targeting constructs using TALEN-based technique (Cermak *et al.* 2011). We successfully introduced a frame-shift deletion in the beginning of the first exon of *nanog* and sox19b and obtained germ line transmission, to F1 generation. *nanog* TALEN microinjection was more than 70 percent efficient and resulted in biallelic *nanog* knockout in some fish. Although TALEN targeting efficiency was not so high for sox19b, we successfully obtained two founder fish, carrying frame-shift mutations in the beginning of sox19b ORF. We are currently raising the F2 homozygous and heterozygous mutant fish to sexual maturity to start the experiments on F3 maternal-zygotic mutants.

The ChIP-on-chip and ChIP-seq

chromatin immunoprecipitation technique allows the genome-wide localization of binding of specific transcription factors or positioning of histone modifications on chromatin. We successfully employed the chromatin immunoprecipitation with subsequent New Generation Sequencing (ChIP-seq) to determine genome-wide binding regions of Pou5f3 and Sox2 in pre- and post-ZGA zebrafish embryos (Leichsenring *et al.* 2013). Currently we are using ChIP-on-chip using Agilent tiling microarrays, designed by us, to map various chromatin epigenetic marks in the wild type and mutant zebrafish embryos in successive developmental stages.

MNase –seq technique

is an enzymatic digestion of native or fixed chromatin in the nucleosome-free regions followed by NGS. This technique makes it possible to determine genome-wide nucleosome positioning, and we use it to investigate if the absence of pluripotency transcription factors leads to nucleosome displacement.

NGS and microarray bioinformatics analysis pipeline

Analysis of genome-wide data represents a significant computational and intellectual challenge. During our previous (Onichtchouk *et al.* 2010; Leichsenring *et al.* 2013) and current work, we developed pipelines for ChIP-seq, MNase-seq, and Agilent tiling and expression microarray normalization and data processing, using commercial and (mostly) non-commercial software (Galaxy workflows, Integrated Genome Browser, etc), which is suitable for use by biologists without prior programming knowledge.

To study the regulation of selected genes, we generate GFP-fluorescent transgenic lines

using Tol2- Gateway transposon-based system (Kwan *et al.* 2007). Tol2- Gateway technique greatly facilitates transgenesis in zebrafish, and allows to combine various regulatory elements, marker protein (GFP), and 3' regulatory sequences using a set of standard entry vectors.

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Molecular Plant Physiology

HEAD Prof. Dr. Klaus Palme

PROJECTS

Fungi-plant communication

Symbiotic interactions between fungi and plants increase dramatically the surface area and therefore the efficiency of roots. We aim to understand the complex network of cues and responses which eventually shape the architecture of the root system, maximizing the mutual benefit to both partner species. We have recently shown that fungal compounds act as potent modifiers of plant growth. In particular, low abundance volatile sesquiterpenes (SQT) stimulate lateral root initiation. Understanding which developmental pathways are requisitioned and how is the research goal of this project.

The auxin efflux complex

Auxin is a major growth regulator in plants. The flow of auxin from its sites of synthesis to its site of action is responsive and finely regulated by the plant. The primary points of control are asymmetrically localized cellular auxin efflux proteins of the PIN family. Our studies show that PINs are components of larger plasma membrane protein complexes. Important questions now centre on how PIN-interacting proteins regulate the rate of auxin efflux (for example through phosphorylation) and effect correct PIN localization. PIN complex components are likely to link auxin transport to other processes, such as hormone signalling balance, vesicle trafficking, and cytoskeleton dynamics.

Single cells to organs

Doubled-haploid plants (DHs) have become an important tool for increasing the efficiency of plant breeding. They are generated by the *in vitro* cultivation of haploid female or male gametophytic plant cells. Most commonly used are microspores, pluripotent stem cells which develop into pollen, the male gametophytes of seed plants. By carefully selecting experimental conditions, these cells become totipotent by cellular reprogramming. Instead of maturing into pollen grains, an alternative developmental pathway is activated which leads to the development of embryos, which after chromosome doubling, give fully homozygous “doubled-haploid” plants. These clonal populations of completely homozygous DH plants are never seen in nature, speeding up plant breeding enormously and making accessible varieties, which were previously unavailable to breeders. Unfortunately, recalcitrance is common: Microspore embryogenesis only works well in a few species of plant. We are currently investigating the mechanisms of reprogramming and totipotency in plant cells with a view to mechanistically understanding the epigenomic regulation of these processes for the improvement of double-haploid plants manufacture.

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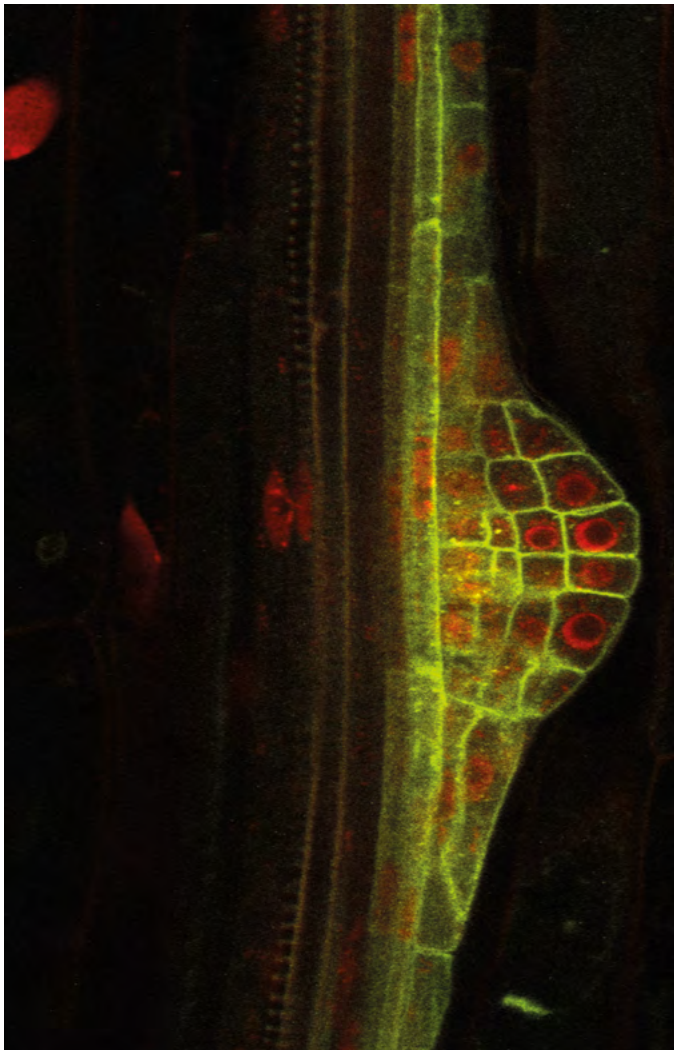

Developing lateral root of an *Arabidopsis thaliana* seedling.
Image: Ditengou, F.A., Palme, K.

METHODS

iRoCS

The computer aided generation of cellular atlases has recently proved very useful in the fields of zoology and medicine, standardizing descriptions of tissues, and underpinning systems biology studies. Our intrinsic *R*oot *C*oordinate *S*ystem (iRoCS) is an anatomical coordinate system which takes three-dimensional images, automatically finds nuclei and cell walls contained inside, and places the extracted data in a biologically relevant context, allowing full cellular annotation of a root apical meristem within a short time interval. With iRoCS, each cell in the root apical meristem is given a co-ordinate, its volume and many other features being recorded. This makes it possible to perform standard statistical tests on root phenotypes at cellular resolution. In addition, each cell is assigned a cell layer and a cell file, making it possible to determine their contributions to growth.

Once trained, the flexibility of iRoCS supplies opens channels with the potential to place any feature which can be captured with a confocal microscope into the root atlas. It is completely genome- and species-independent with no reliance on, for example, GFP tags. Common histological stains are used to highlight desired structures, meaning it can be applied readily to phenotype discovery. iRoCS uses 3D data sets from standard confocal microscopes and is freely available, meaning that it can be widely applied.

Single cell analysis

Protoplasts are single plant cells generated by the removal of their cell wall. As they are easy to transform, uniform, and accessible to chemical treatment, they are ideal tools for a wide range of biological assays and screening applications. The Palme lab has developed a wide variety of such applications. For example, we have developed the protoplast as a platform for a wide range of biological assays, both using fluorescent protein-based sensors, as in the case of a novel auxin assay and a functional amiRNA screening platform, and based on changes in cellular phenotype. To enhance the applicability of protoplast-based screening techniques, automated microscopy coupled with pattern-recognition software afford increases in sensitivity and increase greatly the number of cells which can be screened. Please get in contact if you think using protoplasts or other single plant cells as single-cell test tubes could benefit your research.

Whole mount *in situ* proximity ligation assay (PLA)

Whole-mount *in situ* immunolocalisation has been developed into a well-optimised and reliable technique for the visualization of proteins in *Arabidopsis* seedlings. However, until now, the resolution for detecting co-localizing proteins has been limited by the confines of light microscopy. In cells, protein complexes organize themselves into domains nanometers across. In order to probe the structure of such nanodomains, the Palme lab has developed a technique whereby the proximity ligation assay, an antibody-based method for localizing sites within single cells which contain two target proteins in close proximity, for application in intact roots. By integrating whole mount-PLA with iRoCS, we are able to normalize and overlay populations of roots, giving precise information as to where specific protein-proteins are taking place and how they are being regulated.

For cell and organ analysis a fully robotized high-content imaging pipeline has been developed and is up and running allowing cellular studies over extended periods of time. By this, perturbed cell populations can be studied where individual cells can be quantitatively followed and data used for building generative cell models.

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Neuropathology

HEAD Prof. Dr. Marco Prinz

PROJECTS

Microglia origin, fate, and function

Microglia are tissue-resident macrophages in the central nervous system (CNS). They belong to a group of mononuclear phagocytes that comprises peripheral tissue macrophages, CNS-associated macrophages, dendritic cells, and monocyte-derived cells (Prinz & Priller 2014). As such, they are critical effectors and regulators of changes in CNS homeostasis during development and in health and disease (Prinz *et al.* 2011). All mononuclear cells originate from hematopoietic stem cells (HSCs) and develop along distinct differentiation pathways in response to endogenous and environmental cues. It was assumed that bone marrow-derived monocytes, a subgroup of leukocytes, circulate in the blood and enter the tissues (where they differentiate into tissue-resident macrophages) in non-pathological conditions and during inflammation. However, this view has been markedly changed in recent years as a result of the discovery of new subtypes of mononuclear phagocytes and their distinct roles in CNS disorders. In the past, our lab could identify the peripheral bone marrow-derived precursor that gives rise to microglia during diseases (Mildner *et al.* 2007). Recently, our lab was able to identify the microglia precursor in the yolk sac and could further decipher the transcriptional programme that is required for the development of erythromyeloid precursors to mature microglia during development (Kierdorf *et al.* 2013). Furthermore, our lab established the first inducible microglia-specific Cre mouse for gene targeting in those cells (Goldmann, Wieghofer *et al.* 2013). In sum, our research revealed a pivotal role of microglia for the CNS during health and disease.

Interferons and the brain

The action of type I interferons in the central nervous system (CNS) during autoimmunity is largely unknown. We demonstrated that mice devoid of the broadly expressed type I IFN receptor (IFNAR) developed exacerbated clinical disease in the model of experimental autoimmune encephalomyelitis (EAE) accompanied by a markedly higher inflammation, demyelination, and lethality without shifting the T helper 17 (Th17) or Th1 cell immune responses (Prinz *et al.* 2008). The engagement of IFNAR on neuroectodermal CNS cells had no protective effect. In contrast, absence of IFNAR on myeloid cells led to severe disease with an enhanced effector phase and increased lethality, indicating a distinct protective function of type I IFNs during autoimmune inflammation of the CNS (Prinz *et al.* 2008). The action of cytosolic RIG-I-like helicases (RLHs) in the CNS during autoimmunity is largely unknown. We could recently identify a novel function for RLHs as negative regulators of TH1/TH17 responses in the CNS, demonstrate a protective role of the RLH pathway for brain inflammation, and establish oligonucleotide ligands of RLHs as potential therapeutics for the treatment of multiple sclerosis (Dann *et al.* 2012).

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METHODS

The Cre/loxP technique

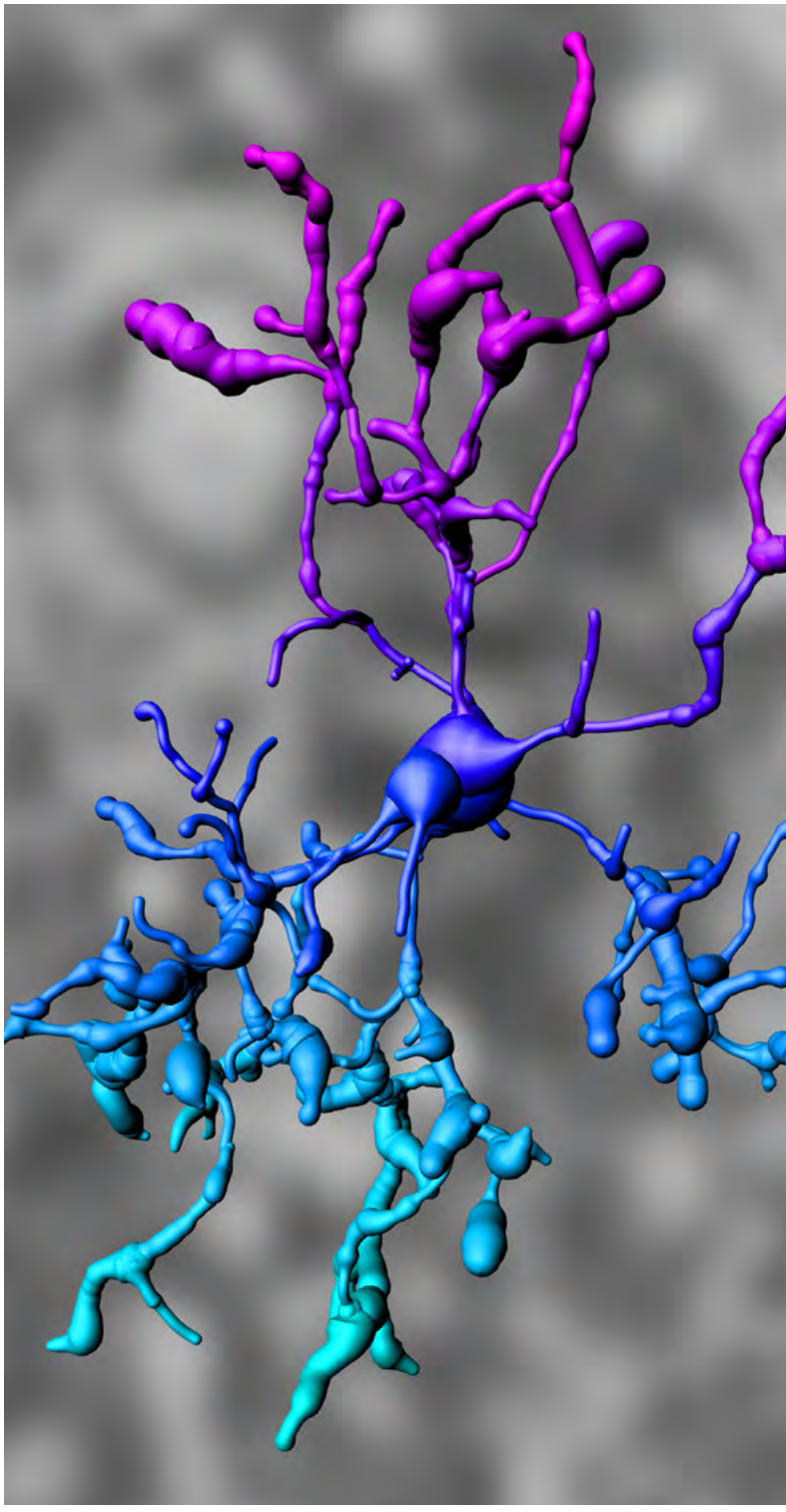
Cre is a DNA recombinase that can recognize and delete so called "floxed" genes, namely genes flanked by LoxP sites. We have previously generated *CX_3CR1-Cre-ER-T2 mice* that harbour a fusion protein between Cre and a mutated estrogen receptor under control of the *Cx3cr1* gene. Consequently, microglia are highly targeted by this mean after administration of tamoxifen due to their high expression of *Cx3cr1*. We crossed these mice to YFP reporter mice and could show that microglia stay constantly labelled whereas other CX_3CR1 expressing peripheral cells, like monocytes, loose the label over time creating confirming the specifity for microglia (Goldmann, Wieghofer *et al.* 2013). Furthermore, by cross-breeding the *CX_3CR1-Cre-ER-T2* mice with *Tak1-floxed* mice we were able to conditionally knockout TAK1 exclusively in microglia which were protected against experimental autoimmune encephalomyelitis (EAE) induction and showed virtually no signs of inflammation and neurodegeneration (Goldmann, Wieghofer *et al.* 2013).

The IMARIS based morphometry technique

This technique allows the quantitative assessment of morphometric parameters of microglia as an indicator of disturbed homeostasis. High-resolution confocal imaging of 30 µm thick brain slices allows to reconstruct the full cell body of ramified microglia. By this method, we could show that the deletion of TAK1 has no consequences for the homeostasis of microglia under steady-state while it has tremendous effects under inflammatory conditions, as shown by a protected phenotype in the EAE model (Goldmann, Wieghofer *et al.* 2013). This method was very valuable to confirm the ability of newly defined yolk sac derived microglia progenitors to develop into ramified microglia *ex vivo* in hippocampal slice cultures (Kierdorf *et al.* 2013). In another study, we investigated the morphometric status of microglia under germ-free conditions where we found a hyperramified phenotype due the disturbed/absent flora in the gut. Therefore, we are the first to show a direct connection between resident immune cells of the CNS and the presence of microbiota in development, health, and disease (Erny, Hrabě de Angelis *et al.* 2015, in press).

Flow Cytometry

The use of multi-channel flow cytometry gives us the possibility to broadly characterize microglia but also peripheral blood cells or other macrophages with high sensitivity on protein level. This method was used to characterize the several transgenic lines that were supposed to be specific for microglia and to compare them against each other. In fact, we could



3D imaris reconstruction. Image: P. Wieghofer

show that our new *CX$_3$CR1-Cre-ER-T2* model is superior to all other models used before with regard to highly targeted microglia with no contribution of peripheral blood monocytes (Goldmann, Wieghofer *et al.* 2013). In another study, we identified microglia precursors in the yolk sac for the first time (Kierdorf *et al.* 2013). We characterized them by their surface marker expression and that they strongly rely on the presence of Pu.1 and *Irf8* in distinct developmental stages (Kierdorf *et al.* 2013). Purification of microglia by flow cytometry from germ-free mice revealed a disturbed expression profile under microbiota-free housing conditions. Identified target genes were shown to mechanistically link the absence of microbiota and the observed phenotype in microgla (Erny, Hrabě de Angelis *et al.* 2015).

Laser Microdissection

This method allows us to dissect single cells from tissues and to purify the RNA for expression studies. The extraction of macrophages from yolk sac macrophages, embryonic microglia, and adult microglia enabled us to identify key factors that are regulated during development.

Electron microscopy

In our study about the origin of microglia, we conducted electron microscopy (EM) to quantify the microglia density in the brain of *Irf8* KO mice. A lack of IRF8 leads to an altered expression of regularly histologically stained markers. The use of EM confirmed our findings gained by immunofluorescence on ultrastructural level (Kierdorf *et al.* 2013).

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Developmental Signalling

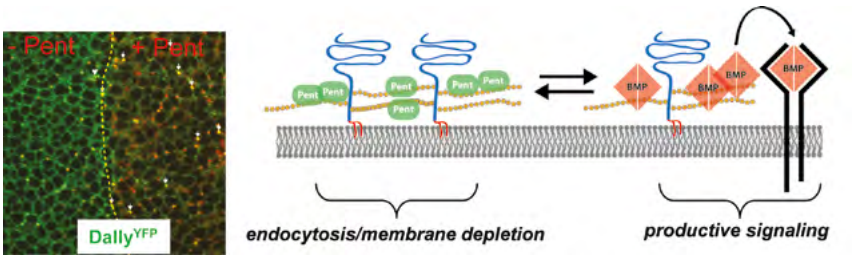
HEAD Dr. Giorgos Pyrowolakis

We have a strong interest in deciphering mechanisms that shape developmental signalling. We are particularly interested in gradients of signalling activities (morphogen gradients) which globally affect groups of cells to coordinate differentiation and growth during organ development. We are using *Drosophila* as a model organism and Bone Morphogenetic Protein (BMP) signalling, a prime example of morphogen signalling in multiple epithelia, to understand how gradients of signalling activity are built, how they are interpreted by the cells of the developing tissues, and how tissue-specific cues impinge on the mechanisms of gradient establishment and/or interpretation.

PROJECTS

Regulation of BMP (morphogen) signalling at the plasma membrane

Many of the regulators of BMP morphogen signalling act (or are predicted to act) at the level of the plasma membrane of signal-receiving cells to affect ligand-receptor interactions or receptor localization and turn-over. We apply a combination of *Drosophila* genetics, quantitative imaging, and novel genomic tools that allow monitoring components of the signalling system and their interactions under physiological levels and conditions to address the role of endocytosis and compartmentalization at the plasma membrane. In addition, we employ newly established, quantitative sensors for BMP signalling activity in RNAi-based screens to identify transmembrane and extracellular proteins that affect BMP gradient formation in the wing imaginal disc.



Compartmentalization of BMP signalling at the plasma membrane: Glypicans, heparan sulfate proteoglycans that are hooked to the cell surface by a GPI-anchor, are critical for BMP signal activation and gradient formation. The secreted protein Pent binds to glypicans and triggers their internalization and membrane depletion. Image: Research group Pyrowolakis

bioss.science/pm/developmental_signalling

Understanding cis-regulatory principles in morphogen gradient interpretation

We have a long standing interest in deciphering transcriptional responses to BMP signalling, particularly in the context of graded BMP signalling. We have identified branches of Smad signalling that differentially affect target gene transcription and have established the cis-regulatory elements and their cognate transcriptional co-factors that implement such responses. We employ the derived sequences motifs to identify novel BMP-targets in genome-wide, *in silico* screens. In addition, we address evolutionary aspects of BMP-dependent responses and processes and the integration of BMP signalling with other developmental signalling pathways, including Notch, WNT, EGFR, and JNK signalling at the level of target gene regulation during normal and aberrant development.

Addressing regulatory feedback *in vivo*

Many of the identified BMP morphogen regulators are controlled in their transcription by BMP signalling itself. In parallel to our efforts in understanding the molecular activity of such regulators, we investigate the *in vivo* significance of their incorporation in regulatory feedback loops. We use genome editing technologies and our extensive knowledge on transcriptional regulation in BMP signalling to modify the strength and polarity of selected regulatory feedback circuits and address their impact on gradient formation and on inherent properties of the system including scaling and robustness. Along the same lines, we investigate whether and how tissue specific cues might rewire feedback regulatory circuitry to alter signalling dynamics and, consequently, signalling output and organ size, shape, and function.

METHODS

Imaging (LSM, live-imaging), ***Drosophila* genetics** (gain- and loss-of-function analysis, epistasis, RNAi-screens, mosaic analysis), **fly-transgenesis**, **molecular biology**, **reporter assays** in flies and cell culture, **protein-protein** and **protein-DNA interaction assays** are routinely used in our lab.

Genome editing

We extensively use Cas9/CRISPR-based genome editing to modify components of BMP signalling in their endogenous loci *in vivo*. Sequences to be modified are replaced by an attP sequence which can be then used as a "landing site" to insert modified versions of the original sequence by site directed attB/phiC31-mediated transgenesis. We use this system to modify both cis-regulatory sequences that control expression of selected feedback regulators of BMP signalling and to modify signalling components (ligands, receptors, co-receptors, regulators) at the protein level. Manipulations include the addition of conventional and fluorescent epitope tags for localization studies, the addition of BiFC- and FRET-partners for assessing interactions under physiological levels, and the modification of the protein sequence for structure-function analyses.

Optogenetics

In collaboration with the Weber group, we aim at establishing systems that convert BMP pathway activation from ligand-dependent to light-dependent. BMP receptor subunits are fused to optogenetic partners (PhyB/PIF, CRY2/CIB1) and light-inducible activation of receptor assembly and signal activation are monitored in reporter assays in *Drosophila* S2-cells. Selected combinations will be transferred into the fly-system using genome editing with the ultimate goal to establish systems that allow for tight spatio-temporal regulation of BMP signalling *in vivo*.

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Degradomics and Cellular Pathomechanisms


1


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HEADS

1. PD Dr. Oliver Schilling
2. Prof. Dr. Thomas Reinheckel

PROJECTS

Proteolysis

Proteolysis is an irreversible post-translational modification that is involved in almost every aspect of cellular fate and behaviour. In addition, extracellular proteases provide cellular control of the extracellular environment. The prominent biological relevance of this enzyme class is reflected by its high representation (~ 2 percent of genes, coding for at least 560 proteases and 160 endogenous protease inhibitors) in the human genome.

In normal tissue homeostasis, the interacting network of proteases and their natural inhibitors maintain a proteolytic balance. During any pathological tissue remodelling process, this balance is disturbed affecting proteases of (at least) three major families, metalloproteases (MMPs, ADAM, and ADAM-TS), serine proteases, and cysteine proteases (cathepsins). In tissue remodelling proteases may degrade or fail to degrade extracellular matrix proteins. However, proteases are also essential for immune cell function in inflammation. For instance, the cathepsins are involved in tumour- and lung metastasis-associated stroma activation and inflammation.

The *laboratory of Dr. Oliver Schilling* combines cutting-edge proteomic technologies with cell-biological approaches to characterize molecular specificity of cysteine proteases in the cellular and organism-wide context. In addition to quantitative and functional proteomics, we employ a diverse array of complementary techniques, including molecular biology, cell culture, protein chemistry, and bioinformatics.

In the *laboratory of Prof. Dr. Thomas Reinheckel* we aim to understand protease regulation and function in physiological processes but also in a variety of diseases. In this regard protease-driven carcinoma progression and metastasis are in the focus of our research. To this end, we develop models for selective inhibition of protease activities thereby interfering with disease progression.

bioss.science/pm/degradomics_cellular_pathomechanisms

METHODS

Protease Mouse Models

Constitutive protease deficient mouse lines for cathepsins B, D, E, H, K, L, S, legumain (AEP), and fibroblast-activation protein (FAP)–α. Conditional mouse models for cathepsins B, D, L, and dipeptidylpeptidase 9 (DPP9). Protease transgenic mouse lines for human cathepsins B, H, L, S, and V. Mice and materials derived from those mouse lines are available upon request.

Cancer mouse models

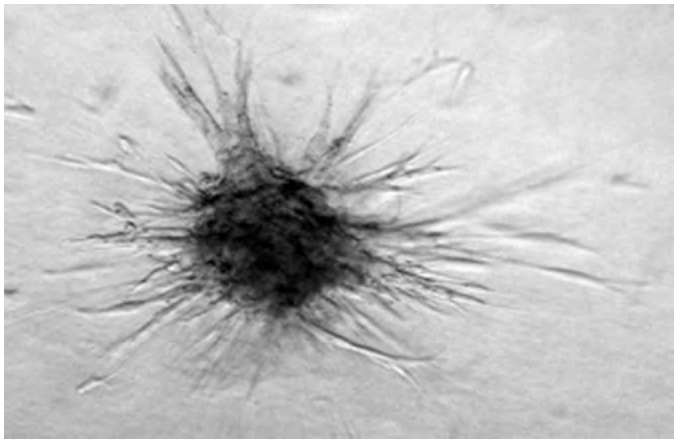
Transgenic MMTV-PyMT model for metastasizing breast cancer as well as K14-HPV16 for skin and cervical cancers. Experimental lung metastasis and orthothopic transplantation models with mouse and human cancer cell lines. Mice and materials derived from those models are available upon request.

The CRISP/Cas9 technique

This technique allows the rapid and efficient deletion or mutation of genes. We employ this technique to alter the genome cancer cells, especially of tumour-initiating cells in order to study proteases and their substrates.

Cell culture in “3D”

In order to study invasive growth of cancers, we frequently use cell assays that mimic the natural microenvironment of cancer cells in terms of extracellular matrix, stroma cells such as macrophages, and added growth factors/cytokines. In those models, cancer cells organize in so called spheroids that further grow into the surrounding extracellular matrix by collective migration/invasion.



Cancer cell spheroid. Photograph by: Research group Reinheckel

Degradomics / terminomics

Proteases generate stable cleavage products with often altered functionality. These proteolytic products are characterized by newly formed N- or C-termini. Recently developed proteomic techniques, termed “degradomics”, allow for their specific analysis and relative quantitation; thus enabling protease substrate discovery and monitoring of proteolytic signalling in biological processes.

Protease specificity profiling

Proteases typically recognize substrates in an extended active-site cleft, often involving multiple interactions between substrate side-chains and corresponding binding pockets of the protease. In many cases, these interactions occur both N- and C-terminally to the scissile peptide bond. Proteome-derived peptide libraries are a powerful tool for the investigation of protease specificity with applications to further areas of enzyme-substrate interaction.

Formalin-fixation, paraffin-embedment (FFPE) represents one of the most widely used archiving strategies for biological specimens. FFPE samples have long been inaccessible to proteomic techniques. We have established robust and sensitive protocols for *quantitative proteomic and degradomic investigations of FFPE specimens*.

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Plant Cell Biology

HEAD Prof. Dr. Stefan Rensing

PROJECTS

Stem cell formation

The moss *P. patens* has a unique capability to reprogram differentiated leaflet cells into apical stem cells upon a combined wounding/osmotic stress trigger. We have analysed the time-resolved transcriptome response of this developmental progression and could thus define key bHLH transcription factors controlling the re-embryonalisation (Busch *et al.* 2013). We could also demonstrate that the homeobox transcription factors of the WOX family are involved in the reprogramming of leaflet cells into stem cells (Sakakibara *et al.* 2014). We try to understand which common principles might guide stem cell development in different groups of organisms.

Epigenetic priming

We developed the first histone code atlas for *P. patens* and analysed it for commonalities and peculiarities (Widiez *et al.* 2014). In general, the activating and deactivating nature of histone H3 marks and their combinations are akin to mammals and flowering plants. In order to determine potential priming responses, we analysed both the developmental progression from juvenile to adult and the effect of drought. We find that the developmental progression primes many genes for their later activation under drought, and that this priming is specific for this kind of stress.

Biotic interactions

Most land plants are able to form mutually beneficial associations with fungi, so-called mycorrhiza. This symbiosis evolved at the time of the water-to-land transition of plant life, around 500 million years ago, at a time when plants did not yet possess roots. We have sequenced and analysed the genomes of such a fungus (Tisserant *et al.* 2013) and of the rootless moss *P. patens*, belonging to a plant lineage that separated from what was to become flowering plants shortly after the evolution of such symbioses. Since the moss genome harbours all genes necessary to engage into such an association (although it is not known to do so in the wild), we reverse engineer mutants that more easily engage with fungi. We study the bipartite signalling taking place prior to and during interaction.

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METHODS

RNA-seq

We are routinely using deep sequencing of cDNA for transcriptomics. We have used several technology platforms and developed *in-house bioinformatics pipelines* to deal with the data processing. In particular, we are able to come up with *reliable lists* of differentially expressed genes and bias Gene Ontology terms, among others.

Comparative genomics

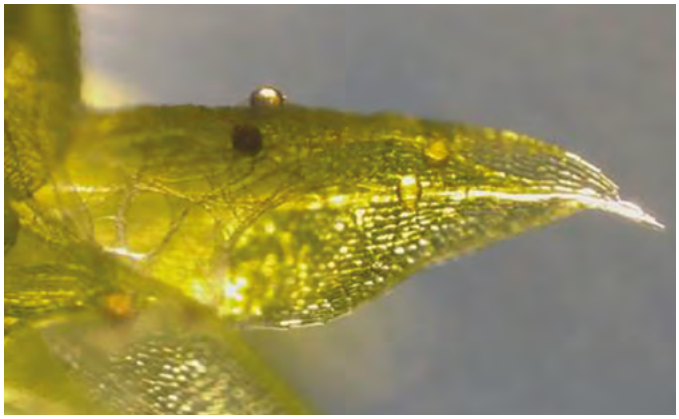
The comparative analysis of transcriptomes and genomes over evolutionary distances is one of our core competencies. In particular, we are able to *reliably annotate transcription factors* and transcriptional regulators, and to *define cis-regulatory elements de novo* (Kreutz *et al.* 2012, Timmerhaus *et al.* 2011).

Phylogeny

Phylogenetic analysis is very powerful for *analysis of orthology* and in order to *understand gene family evolution*. We are able to expertly apply such methods to a variety of datasets in order to come to publishable conclusions. E.g., using such methodology, we could show that mitochondria were already present in the ur-eukaryotic cell, that *Physcomitrella* comprises several cryptic species, how the *light signalling components* COP1/SPA and members of *auxin signalling* evolved, or how *insect and mammalian Neph* proteins and components of the *GARP complex* evolved.

Bioinformatics

All the above methods require skilful use of bioinformatics, which has been established in my group for many years. We have the appropriate *skills, hardware, and software* available to support a vast array of methods.



Spores and hyphae of the mycorrhizal fungus *Rhizophagus irregularis* on leaflets of the moss *Physcomitrella patens*.
Photograph by: Marina Ortega Perez, research group Rensing

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Plant Biotechnology

HEAD Prof. Dr. Ralf Reski

PROJECT

In the project “Glyco-Design at Golgi membranes” the moss-specific N-glycosylation patterns will be modulated towards a humanized form. Our aim is the further flexibilization of the moss *Physcomitrella patens* as a production host for recombinant biopharmaceuticals (moss bioreactor). This will be achieved by the reconstitution of the human sialylation pathway within the moss.

Post-translational modifications like the N-glycosylation in plants are similar to those in humans, but several differences affect product quality and efficacy. Modifications of the plant N-glycosylation pathway are necessary to abolish putatively immunogenic plant-specific sugar residues and in addition to increase *in vivo* activity and serum half-life of the biopharmaceutical product. In the process of glyco-design the activity of endogenous plant-specific glycosyltransferases was prevented. In order to rebuild a humanized N-glycosylation pathway, the introduction of different human enzymes will be necessary. To prevent an incorrect localisation of the human enzymes in the plant Golgi apparatus, synthetic chimeric enzymes will be designed. These enzymes combine the catalytic domain of the human enzymes activity and plant Golgi membrane localization domains to achieve correct organization within the Golgi apparatus. The organization of the glycosylation enzymes in the sequential signalling network will be analysed and N-glycosylation patterns will be examined via mass spectrometry of target proteins.

Detail of a moss bioreactor producing moss-made pharmaceuticals.
Courtesy: Eva Decker, Plant Biotechnology, University of Freiburg

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METHODS

Highly specific gene targeting

A key feature of moss for the flexible adaption of metabolic or signalling pathways is its innate high rate of homologous recombination in vegetative cells, allowing highly efficient precise gene targeting with base-pair specificity. For optimization of the moss bioreactor, gene targeting was used to engineer N-glycosylation, thus abolishing putatively immunogenic plant-specific sugar residues from the biopharmaceutical products. Functionality of genes can be disrupted by targeted knockout approaches. Knockout constructs used for the transfection of moss protoplasts regularly consists of 700 to 1,000 base pairs of genomic DNA flanking each side of a selection cassette, which interrupts or replaces the target gene when indicated.

Glycan analysis via mass spectrometry

Mass spectrometry is a highly specific tool for the analysis of N-glycans and glyco-optimization steps. Analyses are done via electrospray ionization technique by quadrupole-time-of-flight tandem mass spectrometry. Resulting data are analysed by peptide-database research using Mascot Distiller and followed by database reconciliation with all *Physcomitrella patens* protein models according to the central bioinformatics resource www.cosmoss.org developed and hosted by us, and the additional human EPO sequence.

Biopharmaceutical production in photo-bioreactors

Biopharmaceutical-producing moss lines can be cultivated under standardized and controlled conditions in photo-bioreactors. For proof-of-concept studies on productivity of moss lines and bio-activity of the products we employ five litre glass tanks. The moss is stirred by a central rotating impeller. Aeration, light intensity, and temperature as well as pH of the cultures are controlled and can be adjusted according to the needs of a specific line. Taking advantage of several characterized moss-derived signal peptides, the recombinant product will be released to the surrounding medium, thus facilitating the down-stream processing.

Cryopreservation

Moss plants can be cryopreserved for many years over liquid nitrogen in the central resource International Moss Stock Centre (IMSC) Freiburg (www.moss-stock-center.org), developed and hosted by us and co-financed by BIOSS. This facilitates Master Cell Banking according to GMP standards.

Moss methods

Moss-specific methods can be found on our homepage: **www.plant-biotech.net**

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Molecular Immunology

HEAD

❶ Prof. Dr. Michael Reth

PROJECT LEADER

❷ Dr. Jianying Yang


❶


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Photograph: Max Planck Institute of Immunobiology and Epigenetics

PROJECTS

B cell activation

Our group works on signalling processes involved in development and activation of B lymphocytes. In particular, we study the proliferation to differentiation switch in developing B cells as a dysregulation of this process can cause immunodeficiencies and tumours, such as leukemia. All mature B lymphocytes carry a B cell antigen receptor (BCR) on their surface. We use super-resolution techniques as well as synthetic biology approaches to better understand the nanoscale conformation and signalling mechanism of this receptor. Specifically, we found that on resting B cells the BCR forms auto-inhibited oligomers that are opened upon receptor activation.

The kinases/phosphatases equilibrium

The spleen tyrosine kinase Syk and the protein tyrosine phosphatases SHP-1 are regulating signalling output from the BCR. The first signalling event is the phosphorylation, binding, and opening of the BCR by Syk. The formed BCR/Syk complex not only activates several downstream signalling pathways but also phosphorylates inhibitory receptors and activates SHP-1. The phosphatase then dephosphorylates the BCR and thus dissociates the BCR/Syk complex. Our recent studies show that this kinases/phosphatases equilibrium is not only important for normal B cell development but also plays an essential role in the establishment of tumours such as acute lymphatic leukemia.

The resting BCR

Even though there exist many studies on the signalling function of the BCR on activated B lymphocytes, hardly anything is known about the conformation and regulation of the BCR on resting B cells. In our super-resolution studies we found that on resting B cells the BCR form specific oligomers that are residing inside 80 to 150 nm large protein islands, whose composition we are studying with new proximity proteomics techniques. We also found that the resting BCR is methylated by the protein arginine methyl-transferase 1 (PRMT1) and associated with the cytoskeleton. Specifically, the inhibition of the actin polymerization results in BCR opening and activation. To identify the cytoskeleton elements that guard the resting BCR, we use proximity proteomics approaches. The identified elements are further characterized by loss-of-function (Cre/loxP and CRISP/Cas9) and gain-of-function (S2 system) methods.

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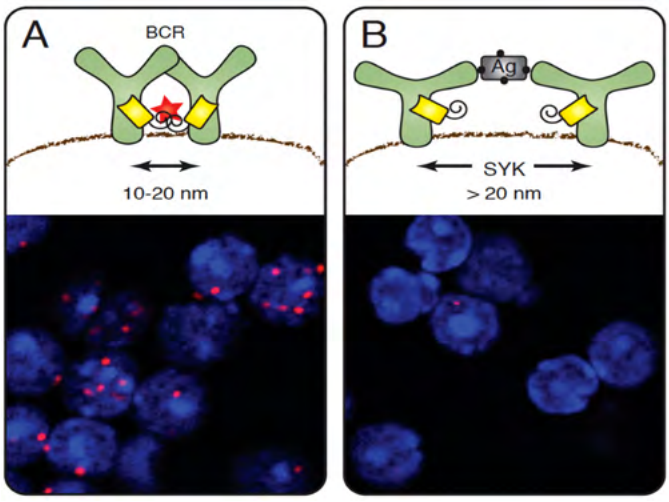
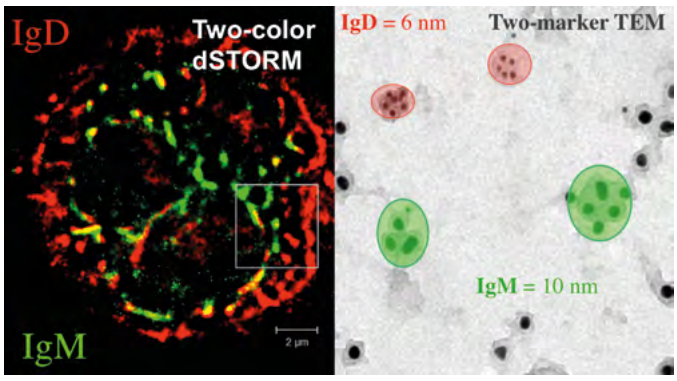


The Fab-based proximity ligation assay (Fab-PLA) measures the conformation of the B cell antigen receptor (BCR) at 10-20 nm distances. For this, Fab fragments from anti-BCR antibodies are coupled with oligos that drive an amplification reaction when they are closer than 9 nm together. A: Scheme of the conformation of the BCR on resting B cells and the result of the Fab-PLA assay. B: Binding of antigen (Ag) to the BCR results in receptor dissociation and thus in the loss of the PLA signal. The dissociation is also mediated by binding of the spleen tyrosine kinase (Syk) to the BCR.
Image: Research group Reth



Mature B cells co-express two types of BCR on their cell surface, namely (green) the IgM-BCR and (red) the IgD-BCR. Left picture: The two-color dSTORM analysis shows that on the surface of resting B cells the two receptor classes are distributed inside separated protein islands. Right picture: Analysis of the distribution of the two BCR classes by two-marker transmission electron microscopy (TEM). The different classes of receptors are connected to different peptide Tags allowing their detection by anti-Tag antibodies that are coupled to either 6 nm gold particles (IgD) or 10 nm gold particles (IgM). Image: Research group Reth

METHODS

The Cre/loxP technique

Cre is a DNA recombinase that can recognize and delete so called “floxed” genes, namely genes flanked by LoxP sites. We have previously generated MerCreMer, a fusion protein between Cre and two Tamoxifen-binding Mer domains and shown that the MerCreMer enzyme only becomes active in the presence of Tamoxifen. The MerCreMer system is a useful genetic tool to activate or delete genes in the genome of a living organism (see Busch *et al.* 2015). We have also generated knock-in mice carrying cDNA of either Cre, MerCreMer, or CreERT2 (another Tamoxifen-regulated Cre) inserted into the mb1 gene. With these mice one can delete genes in a B-cell-specific and inducible manner as recently demonstrated for the Syk gene (Hobeika *et al.* 2015). All these Cre mice are available at the Reth lab.

The CRISP/Cas9 technique

This technique allows the rapid and efficient deletion or mutation of genes. We employ this technique to alter the genome of the human B cell line Ramos and we are generating many signalling mutants of these cells. All our CRISP/Cas9 targeting vectors are available at the BIOSS Toolbox.

The S2 *Drosophila* Schneider cell rebuilding system

To counteract the dominance of the loss-of-function and knock-out techniques in biological research we are promoting gain-of-function approaches. We have thus developed a method allowing the transient and inducible co-expression of up to twelve genes in S2 *Drosophila* cells. With this synthetic biology approach one can first rebuild a given signalling pathway and then start to modify and alter it in several ways. This allows a deeper understanding of signalling mechanisms and the role of each component of a given signalling pathway. With this method we discovered that the kinase Syk opens and activates the BCR via a novel inside-out signalling mechanism.

Super-resolution techniques

Due to diffraction barrier of the light microscope at 250 nm, little is known about the organization of receptors on the plasma membrane at 10 to 200 nanometers (nm) distances. We have established several techniques that can monitor the relative location of membrane proteins at the range of 10-80 nm. One of these methods is the *in situ* proximity ligation assay (PLA) involving DNA-oligo-coupled antibodies specific for two target proteins, a rolling circle amplification and fluorescence-coupled oligonucleotides for detection (Soderberg *et al.* 2008). By using oligo-coupled Fab fragments we have improved the detection to the 10 to 20 nm range (Kläsener *et al.* 2014). This improved *Fab-PLA* method allowed us to study for the first time

the dissociation of the BCR upon exposure to antigen *(see first picture)*. In our nanoscale studies we also employ the *direct stochastic optical reconstruction microscopy (dSTORM)*. Specifically, using two different colour channels of the total internal reflection fluorescence (TIRF) field with an ELYRA PS.1 microscope (Carl-Zeiss Microscopy, Munich, Germany) we have obtained *two-color dSTORM* images demonstrating the separated location of the IgM-BCR and IgD-BCR (Maity *et al.* 2015). These findings of our super-resolution analysis were confirmed by a two-marker transmission electron microscopy *(TEM)* study *(see second picture)*.

Sortase technology

To label small proteins with fluorescent groups (Y) or DNA oligos (see PLA), we are employing a transpeptidation reaction of the staphylococcal sortase. For this we express small proteins such as nanobodies or single-chain antibody fragments carrying the LPXTG sortase-recognition motif at their C-terminus. The sortase cleaves the motif between the threonine and glycine forming an acyl-enzyme intermediate. The N-terminal amino group of the GGG-Y resolves the intermediate by a nucleophilic attack to generate an LPXT-GGG-Y fusion protein. Many reagents for the sortase reaction are available at the BIOSS Toolbox.

Aptamer technology

Aptamers are small 25 to 70 bp large single-stranded DNA or RNA molecules with binding specificity against a given molecule (protein or chemicals). It is thought that in the future aptamers will replace the monoclonal antibody technology. Specific aptamers can be isolated from a large (10^{15}) library via the "selection of ligands by exponential enrichment" *(SELEX)* procedure. We are currently generating aptamers against different isotypes of the BCR and B cell coreceptors. Once isolated, aptamers can be easily produced in large amounts and combined with fluorescent groups or PLA oligos for super-resolution studies.

Proximity proteomics

Signalling processes occur at localised privileged sites on or inside cells. To learn more about the nano-environment of these signalling hubs, we are employing proximity proteomics approaches. One method known as *BioID* employs the promiscuous biotin ligase mutant (BirA*) to biotinylate proteins in the vicinity of a signalling component X. For this we express X-BirA* fusion protein in wild type or X-deficient cells in the absence of biotin. At different exposure times to biotin the cells are lysed and the biotinylated proteins purified by Streptavidin and identified by mass spectrometry. Other, alternative next-neighbour-labelling methods are under development.

BiFC

In an attempt to prove that the BCR form spontaneously dimers in living cells, we have adapted the bifluorescence complementation *(BiFC)* method (Yang and Reth 2010). We fused the half-domains of YFP and CFP (YN and CC) to BCR components and expressed them together in *Drosophila* S2 cells. The fluorescence signal from the reconstructed YFP then allowed us to monitor the oligomerization of the BCR on living cells. Furthermore, we developed a new method based on the immunoprecipitation of multiprotein complexes detected by flow cytometry *(IP-FCM)* to quantify the amount of fluorescence generated. This study showed that the formation of oligomers is an intrinsic feature of the BCR. All BiFC constructs are available at the BIOSS Toolbox.

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Photograph: Sebastian Bender

Bio- and Nano-Photonics

HEAD Prof. Dr. Alexander Rohrbach

PROJECTS

Mechanics is the science of moving bodies driven by forces, biology is the science of living organisms. The basic living systems are cells, which are in steady motion driven by a variety of forces out of equilibrium and are regulated by a manifold of signalling events. All these forces are based on physical principles. The investigation of fast dynamic processes in living cells resulting from these forces requires fast measurement technologies – faster and more precise than most technologies which are currently commercially available. This is where we do the main part of our research: We develop fast optical measurement and manipulation technologies and thereby we investigate fast processes down to a molecular scale both in eukaryotic cells and bacteria as well as in biomimetic systems.

OUR BIOLOGICAL PROJECTS

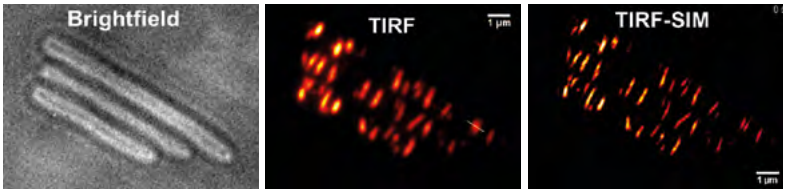
- The nanomechanics of phagocytosis and in filopodia
- The nanomechanics of induced particle uptake in giant unilamellar vesicles
- The nanomechanics of fast deforming helical bacteria (Spiroplasma)
- The dynamics of the cytoskeleton filament MreB during bacterial cell wall synthesis
- The nanomechanical properties of microtubuli

bioss.science/pm/bio-_and_nano-photonics

METHODS

A. High contrast superresolution microscopy at 1 to 10Hz using structured illumination and TIR-fluorescence[1]

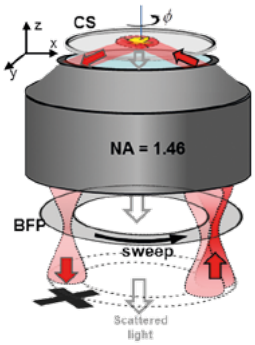
We use a combination of structured illumination microscopy and total internal reflection fluorescence microscopy, where nine sinusoidally modulated raw images are combined to one final superresolved image with 100 nm resolution inside living cells. The current set up achieves a final frame rate of 1 Hz, the new setup is supposed to achieve 10 Hz. The superior image quality inside living B. Subtilis bacteria can be seen in the following images.

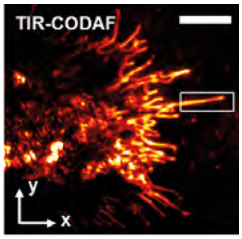


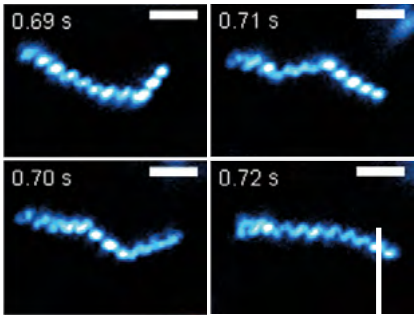
All images:
Research group Rohrbach

B. Label-free high contrast superresolution microscopy at 100Hz using coherent dark field illumination in TIR mode (TIR-CODAF)[2]

This new technique uses a laser beam that illuminates a living, unlabelled cell under an oblique angle as illustrated in the following figure. Laser light is multiply scattered at the cellular structures and a distribution full of speckles is obtained that does not resemble a cell much. However, by sweeping the laser beam along a circular path (see figure) during the integration time of the camera (e.g. only some milliseconds), all the speckles cancel out and a high contrast image of the cell is obtained, as shown by an unlabelled J774 mouse macrophage displayed in the following figure (centre). We call this new technique “total internal reflection coherent dark field (TIR-CODAF) microscopy”. It achieves a resolution of 150 nm at a frame rate of 100 Hz (see 4 images of kinking Spirosplasma on the right) and enables to require many thousands of images without visible loss of image quality.

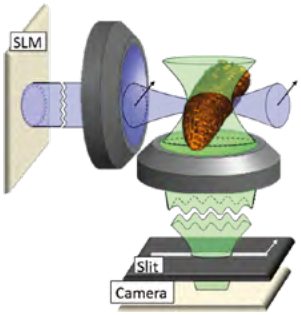


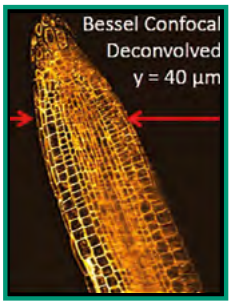




C. Light-sheet microscopy using confocal Bessel beams [3/4]

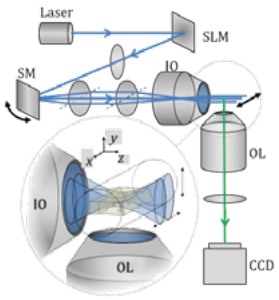
Light-sheet microscopy is mainly applied to large fluorescence labelled objects and has the advantage that it only illuminates that part of the object that is in the focal plane of the detection lens. Thereby, it does not only reduce photobleaching, but is also much faster than 3-D imaging techniques such as confocal microscopy. Using Bessel beams is the illumination beams in combination with confocal slit detection, resolution and contrast can be further increased relative to standard Gaussian beams and image artifacts such as stripes can strongly reduced. The following images show the microscopy principle and a strongly scattering root tip of 150 µm diameter, which has been recorded from only a single direction.

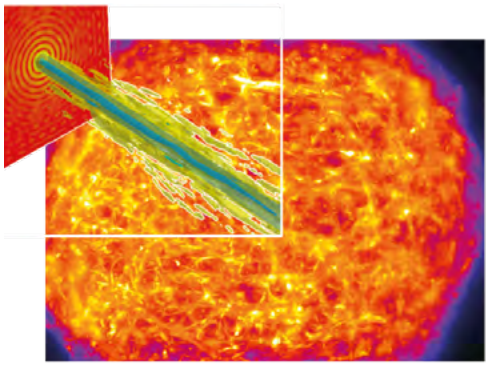




D. Nonlinear light-sheet microscopy using two-photon fluorescence excitation [5]

Using Bessel beams in combination with a pulsed NIR laser beam to enable two-photon fluorescence excitation allows to suppress the fluorescence from the Bessel beams ring system. Thereby one can generate a very thin needle of light with superior propagation properties. Scanning this light needle laterally results in a very thin light-sheet with minimal aberrations due to scattering inside the object. The following figure shows a 250 µm large cancer cell spheroid with actin labelling recorded from a single direction. The penetration depth of the NIR Bessel beam was 550 µm.





E. Dynamic optical traps to induce unlikely biological events[6/7] and

F. Nanometer precise 3D interferometric particle tracking at 1MHz to read out molecular fluctuations[6/7]

This method bases on optical trapping and ultra fast 3-D tracking of small particles and can be combined with any common microscopy technique. Here, a spherical particle, either an uncoated or a specifically coated bead, is positioned by an optical trap to a place of interest, as e.g. the surface of a thin filopodium as illustrated in the figure below. Here, the Brownian motion of the bead inside the optical trap, the contacting and binding to the membrane of the filopodium, various stop and go transport processes can be analysed on a molecular scale by the interferometric particle tracking using scattered laser light. By extracting the transport velocities, the on- and off-binding times, the changes in binding stiffness and viscosity from the particle trajectories in x,y, and z direction (FIGURE 2b), several molecules involved can be identified or excluded to a certain extent without fluorescence labelling. The forces controlled by the optical trap represent an excellent tool for defined and strongly localized mechanical (and chemical) stimuli.

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Synthetic Biology of Signalling Processes

HEAD Jun.-Prof. Dr. Winfried Römer

PROJECTS

The various roles of lectins in bacterial infection

Our research team studies the interactions of human pathogens and pathogenic products with human cell lines of lung, intestine, brain, kidney, and the immune system by using a highly interdisciplinary research approach at the interface of biology, medicine, physics, and chemistry. For instance, highly specific interactions of carbohydrate-binding proteins (called lectins) with distinct host cell glycosphingolipids lead to the formation of plasma membrane invaginations, activation of signalling molecules, cytoskeleton remodelling, and cellular uptake of the pathogen. Moreover, we aim at identifying microbial and host cell factors that are crucial for pathogenic uptake by applying biochemical, screening, and OMICS approaches and at designing small molecule inhibitors towards them. For *in vitro* reconstitution of cellular processes, we develop a unique platform for membrane nanoscopy based on natural and synthetic membrane systems of different complexity in combination with super-resolution fluorescence and atomic force microscopy.

METHODS

Synthetic membrane systems

Synthetic membrane systems, such as giant unilamellar vesicles and solid supported lipid bilayers, have widened our understanding of biological processes occurring at or through membranes. Starting with a minimal system, which is easy to understand, different parameters can be integrated step by step in order to increase complexity in a highly controlled manner. Artificial membrane systems are particularly suited to study the inherent properties of membranes with regard to their components and characteristics. Transmembrane-spanning proteins and protein complexes can be reconstituted in small proteo-liposomes, which then will be fused with giant unilamellar vesicles in order to be able to observe membrane dynamics by fluorescence microscopy. Furthermore, cluster sizes, distribution, and topography can be extracted from data acquired by atomic force microscopy.

Fluorescence microscopy techniques

Laser scanning confocal with a high resolution Galvano mode or an ultrafast resonance scanning mode is routinely used to study interactions of lectins with native cells or synthetic membrane systems. In order to study lectin-induced cellular rearrangements at length scales below the diffraction limit (~250 nm), the superresolution method STORM (stochastic optical reconstruction microscopy) is employed. The aim is to study lectin-induced micro- or nano-clustering of cellular receptors or signalling molecules, which initiates various

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signalling cascades or simply leads to endocytosis of the lectins. In the end, two-colour superresolution will be used to investigate co-localizations of lectins with key structures of the cell membrane on the nanoscale.

Atomic force microscopy (AFM)

AFM is employed as a complementary imaging technique, which provides nm-resolution under physiological conditions. Supported lipid bilayers are imaged via AFM to study the interaction of bacterial lectins with glycosphingolipids and the impact on membrane micro/nano-domains. Furthermore, AFM is used to visualize bacteria-induced rearrangements of the host cell plasma membrane during bacterial attachment and invasion. Here, AFM also allows to directly determine the nanomechanical properties of the sample surface, for example the local stiffness. For evaluation of the role of various cellular key players in the bacterial invasion process, AFM can be combined with conventional fluorescence microscopy or even with the optical superresolution method STORM.

Furthermore, AFM is utilized to measure the elasticity of medically or biologically relevant samples, such as organotypic collagen tissues or photo-switchable hydrogels. A future goal is to quantify lectin-receptor interaction strengths on a single-molecule level by attaching lectins to the AFM tip and measuring the unbinding force from surface-attached receptors.

Synchronization of protein traffic

Our system enables to accumulate a protein of interest at an intracellular compartment from which the protein can be instantaneously released upon addition of a membrane permeable small molecule. This synchronization of trafficking allows imaging and quantifying of individual trafficking steps that are impossible to resolve otherwise. The controlled accumulation is enabled by conditional aggregation domains (CADs) that are fused to the protein of interest. After release, endogenous enzymes remove the CADs to avoid further interference. We optimized the system so that only a single cloning and transfection step is required to achieve synchronization. We have successfully applied the method for real-time imaging of protein trafficking in polarized epithelial cells, but many other applications are possible.

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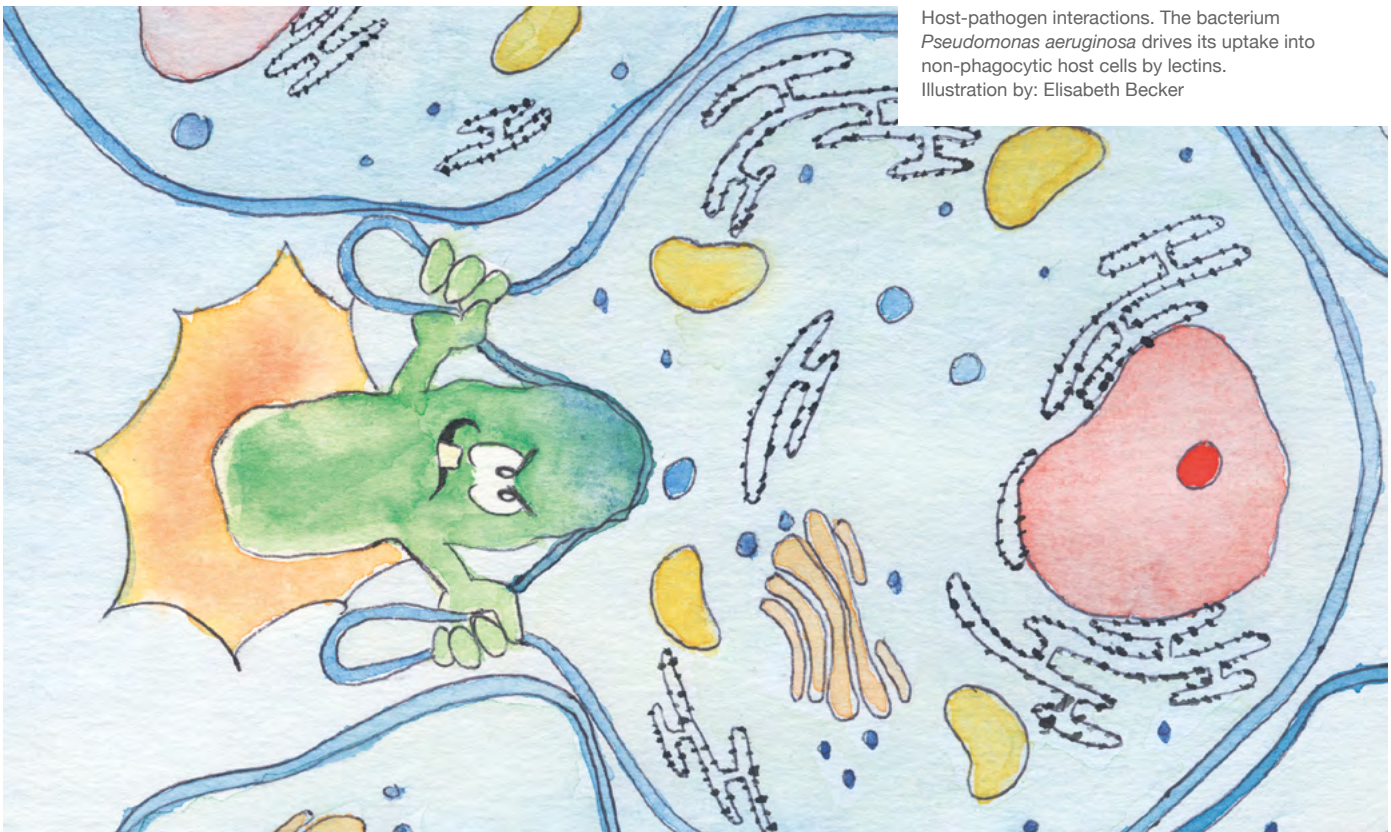

Host-pathogen interactions. The bacterium *Pseudomonas aeruginosa* drives its uptake into non-phagocytic host cells by lectins. Illustration by: Elisabeth Becker

Function of Ribosome-Bound Protein Biogenesis Factors

HEAD Prof. Dr. Sabine Rospert

PROJECTS

Newly synthesized polypeptides pass through a tunnel in the large ribosomal subunit before they are released into the cytosol or into the lumen of the ER. Upon arrival at the exit of the tunnel, the nascent polypeptides encounter a set of proteins, which are essential for subsequent steps of protein biogenesis and are therefore termed ribosome-associated protein biogenesis factors (RPBs, Raue *et al.* 2007). RPBs introduce covalent modifications, mediate targeting to intracellular compartments, and assist *de novo* folding of newly synthesized polypeptides. Moreover, RPBs enable the ribosome to regulate, for example, the rate of translation or translational fidelity in response to specific properties of nascent chains. Some RPBs possess extra-ribosomal functions, which are not directly coupled to the early steps of protein biogenesis. The orchestration of RPBs in time and space is only poorly understood.

We are interested in the ribosomal and extra-ribosomal functions of eukaryotic RPBs. As a model organism we use the yeast *Saccharomyces cerevisiae*. We have expertise in a broad spectrum of methods in yeast genetics and general biochemistry. Many of the techniques can be readily adapted to other model organisms.

METHODS

Ribosome profile analysis

Basic principle: Total cell extracts are loaded onto sucrose density gradients, which separate cytosolic proteins from ribosomal particles. Moreover, ribosomal particles, i.e. small ribosomal subunit, large ribosomal subunit, monosomes, and polyribosomes are separated from each other. Subsequent fractionation of the gradients allows to monitor, isolate, and quantify ribosomal particles and proteins and mRNA molecules associated with them.

What can I do with it? Knowledge of which RPBs bind to the different ribosomal particles is a prerequisite to understand the regulation of protein synthesis, folding, and cellular targeting. Moreover, the ratio between monosomes and polysomes, which can be quantitatively assessed via ribosome profile analysis is an indicator for the cellular energy status. Abnormal ratios of ribosomal particles under specific growth conditions can be an indicator for pathological changes of cellular physiology. Examination of transcripts bound to polyribosomes allows to identify mRNAs, which are selectively translated, or excluded from translation, under specific conditions.

Literature: (Esposito *et al.* 2010; Chiabudini *et al.* 2014)

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In vitro translation of radiolabelled proteins

Basic principle: Translationally active eukaryotic cell extracts can be depleted from endogenous mRNA and can be subsequently complemented with mRNA templates of the experimentalists´ choice. Incorporation of radiolabelled amino acids allows to sensitively follow proteins derived from *in vitro* translation in subsequent experiments. Home-made yeast translation extracts can be employed as a source for translation reactions.

What can I do with it? In vitro synthesized proteins are widely used as a tool to study targeting to, and translocation across cellular membranes. The method allows fast and easy synthesis of small amounts of radiolabelled proteins, which can be used to study interactions with potential partner proteins. *In vitro* protein synthesis can be employed to introduce modified amino acids, for example, to perform site-specific crosslinking experiments. Translation, targeting, folding, or protein-protein interactions can be analysed in the presence or absence of components of interest, because translation extracts can be generated not only from wild type, but also from mutant yeast strains.

Literature: (Garcia *et al.* 1991; Berndt *et al.* 2009; Karamyshev *et al.* 2014)

Analysis of protein phosphorylation via Phos-tag technology

Basic principle: Phos-tag gels provide a means to separate proteins, which differ in their phosphorylation status, but are otherwise identical.

What can I do with it? Kinases and phosphatases regulate function, localization, and protein-protein interaction of their target proteins via addition and removal of phosphate moieties. If a specific antibody for the protein of interest is available, the Phos-tag technique allows distinguishing between unphosphorylated and phosphorylated states of a single protein in total cell extracts. Of advantage, if multiple phosphorylation states exist, one can quantitatively assess their relative abundance. Because the method allows for the parallel analysis of multiple samples it can be employed to analyse the phosphorylation status of a protein under a variety of physiological conditions, in time course experiments, or in a selection of mutant strains.

Literature: (Kinoshita *et al.* 2015)

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Photograph: Philipp Meyer

Microarray Copying and Microcontact Printing

HEAD Dr. Günter Roth

PROJECTS

Our research group develops *methods for real-time label-free detection of molecular interactions* (see first picture) for analysis of molecular interactions. Such interactions can be analysed either in form of a microarray (see second picture) [1] or a micropattern (see third picture) [2] on the surface.

The project ReelinsSys is focussing onto Reelin [2] – a central molecule in neuronal signalling and development. Reelin changes in concentration, isotypes, or mutations may lead to severe neuropsychiatric disorders. As many players of the Reelin-network are only “suspects”, our task is to test these molecules for interaction with Reelin or known Reelin interactors. Therefore, we intend to copy several major biomolecular players from the Reelin pathway onto and into a microarray and to analyse the dynamics and the interaction kinetics of these pathway molecules with each other. Resulting Kd-values, on- and off-rates will generate an additional level of information enabling better simulations for Reelin pathway behaviour and dynamics.

The project Protein-Translator is a more technological focused project. The device contains a unit enabling label-free detection (first picture) capable to make a microarray readout in an imaging real-time mode (https://www.youtube.com/watch?v=tugTLtJaUwQ). We implement and improve microfluidics to generate microarrays via a copying process with up to 100,000 spots of DNA [1] or protein [3]. At the moment this goal for DNA is reached (second picture), for the proteins we are working on upscaling. Also, interaction analysis and Kd-value calculations for other groups are very welcome as it helps us to get our system more into application.

METHODS

The microarrays, which we can readout label-free and in realtime, can be either generated “classically” (via spotting/printing techniques) or via our own novel microarray copying approaches. The copying approach allows to generate DNA microarrays with up to 100,000 spots, up to 1,500 nt long DNA, and free selectable as double or single strand DNA. The copying technique is developed now to the level of DNA-aptamers (second picture) and also to proteins. The output of a copy is simply defined by the enzymatic mix used, DNA- or RNA-polymerase for DNA and accordingly RNA-copy and cell-free mix for protein copies.

bioss.science/pm/microarray_copying_microcontact_printing

The DNA copying technique is now in first application tests with *DNA aptamers* together with Dr. Johannes Kaiser. Also, the *label-free detection of molecular interactions* is currently being discussed with Jun.-Prof. Dr. Winfried Römer to bring his publication (A LecA Ligand Identified from a Galactoside-Conjugate Array Inhibits Host Cell Invasion by Pseudomonas aeruginosa, Angewandte Chemie, 2014) onto the next level by enabling real-time measurement of the binding kinetics as well as competition experiments of the yet identified binders.

In a special workflow, it is possible to copy the DNA of a virus onto our surfaces and subsequently make a protein copy, yielding all proteins of the pathogen. If a blood sample of an immunized individual is flushed over such a copied protein microarray, antibody binding reveals all immunogenic proteins – potential vaccine candidates. We hope to find such a vaccine candidate within two days – due to this, we call this approach "immune2day".

FreiGEM 2015, Freiburgs IGEM-team in 2015, joined us, copied proteins from known pathogens onto surfaces, and made a first proof of concept: They have been able to detect the antibodies against Tetanus toxine in vaccinated persons.

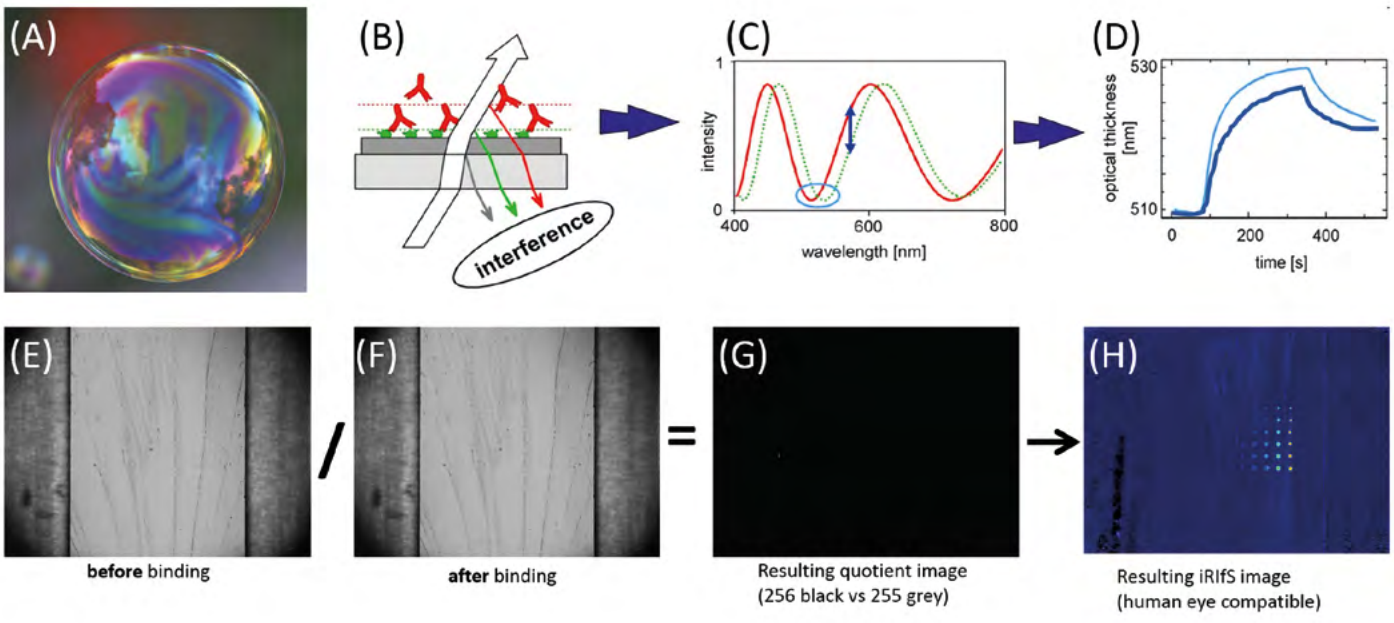
As additional technique, we provide microcontact printing (fig 3). With this technique we can generate a defined molecular environment to the surface as microstructured molecular monolayer. The structure can be generated from proteins, peptides, DNA, antibodies, or interaction-partners such as Reelin (Reelin signals through apolipoprotein E receptor 2 and Cdc42 to increase growth cone motility and filopodia formation. Journal of Neuroscience 2010), or be applied for T-cell stimulation assays (A Peptide-functionalized polymer as a minimal scaffold protein to enhance cluster formation in early T cell signal transduction. Chembiochem 2015). The according structures can be even smaller than the size of a cell and thus enable the identification of sub-cellular structuring of cell-membrane signalling (A Quantitative Assessment of Costimulation and Phosphatase Activity on Microclusters in Early T Cell Signalling. PLOS ONE 2014).

The group has also expertise in microfluidics and assay development as the group leader Dr. Günter Roth was group leader for assay development and assay implementation at IMTEK and involved in the pathway assembler (BIOSS 1) as well as the initial planning of microfluidic PLA assays for BIOSS 2 proposal.

Label-free iRIf-detection for real-time binding kinetics

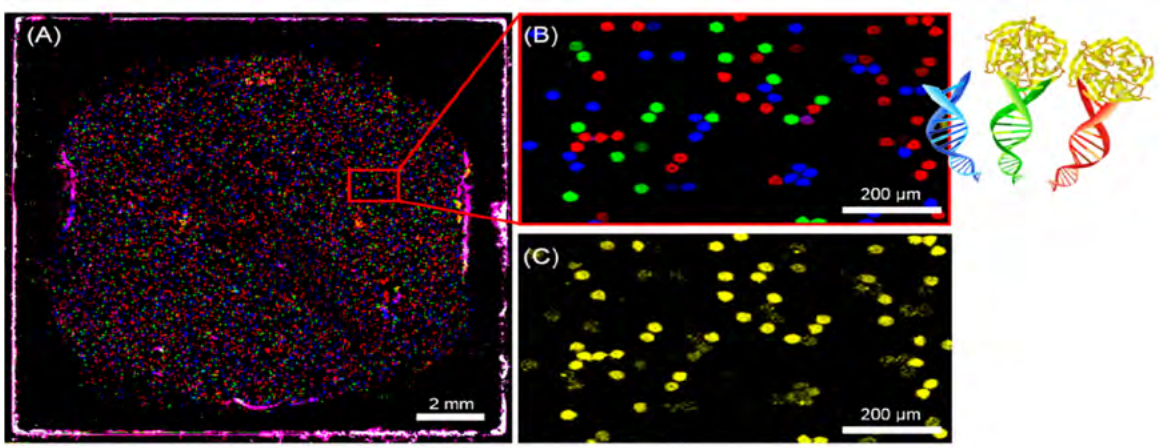
RIfS (Relfectometric Interference Spectrocopy) from the group of Prof. Dr. Günter Gauglitz in Tübingen enables the label-free realtime detection of molecular interactions and Kd-value measurements. We built up several iRIF devices in our lab. iRIF, short for Imaging Reflectometric Interferometry, generates the colour play on soap bubbles (A), and can be transferred to surfaces (B). The change of spectra (C), can be quantified as binding curve (D). The basic mathematics divides the image before binding (E) with the image after (F) and generates a grey value (G) as well as false-colour image (H). Have a look at some binding videos at:

bioss.science/pm/roth-binding-videos



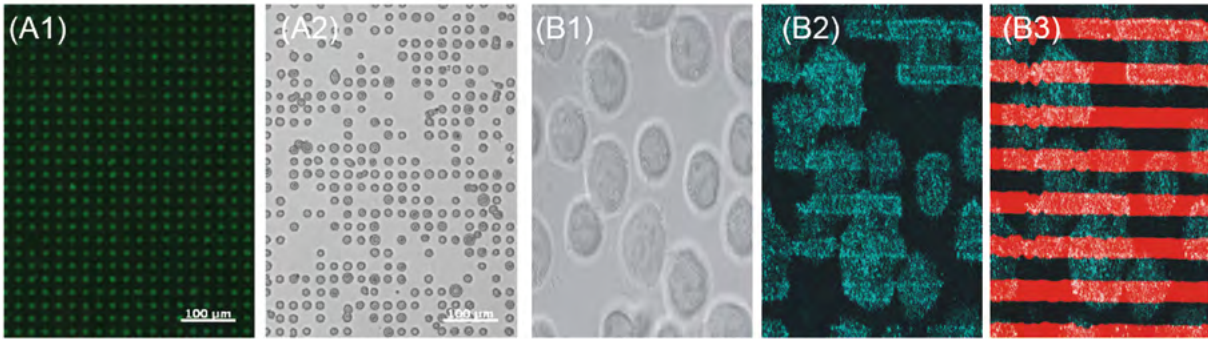
Microarray copying

We can copy DNA directly from solutions onto surfaces. The process is digital, meaning that each spot (A) is generated from one single DNA strand (up to 1500 bp realized). Each dot can be used for hybridization (B) or binding experiments (C).



Microcontact printing (µCP)

We can stamp molecular patterns onto surfaces for cellular experiments (A) and even sub-cellular activation of signalling (B) is possible.



Images: Research group Roth

Membraneprotein Biochemistry and Robotics

HEAD

❶ Prof. Dr. Wolfgang Schamel

PROJECT LEADER

❷ Dr. Susana Minguet

Photograph: Chris Stock-Müller

PROJECTS

TCR nanoclustering

Antigen-independent T-cell antigen receptor (TCR) clustering regulates T-cell sensitivity, as it enhances the avidity of the TCR-antigen interaction[1]. We have previously shown that the nanocluster formation is dependent on cholesterol and sphingomyelin, and that the nanoclusters of the resting TCR localise in the non-raft compartment[2]. We also found that cholesterol specifically binds to the TCRβ chain *in vivo*. We proposed that the sensitivity of memory as compared to naive T cells is increased due to lipid-induced TCR nanoclustering that enhances the avidity to the antigen[2/3].

CD3 conformational change

In an effort to study the earliest events of TCR triggering, we have shown that in unstimulated αβ-T cells the TCRs exist in a closed, inactive conformation, in which a proline-rich sequence (PRS) within the cytoplasmic tail of CD3 is hidden. Upon ligand binding, a conformational change (CD3 CC) is induced exposing the PRS, and thus allowing the binding of the adaptor protein, Nck[4]. In contrast to the αβTCR, in which the CD3 CC is required for TCR activation, antigen stimulation of the γδTCR does not induce the CD3 CC, nor does γδTCR activation require it[5]. However, treatment of Vγ9Vδ2 γδ T cells with the monoclonal anti-CD3 antibody UCHT1 results in enforced CD3 CC, exposes the PRS, and leads to increased *in vitro* tumour cell lysis[5]. In our most recent study, we show that cholesterol binding to the TCRβ transmembrane region can control the shift between the open and the closed states of the TCR. These results present a mechanism by which lipid-receptor interactions can regulate signal transduction.

Kinetic control of T-cell activation

T cells are stimulated by repetitions of short intervals of peptide-MHC binding to the TCR and pauses in between, but it is not understood, how these oscillatory inputs are interpreted by the intracellular signalling pathways of the T cells. To mimic these oscillatory TCR stimulations, we have engineered an optogenetic system in which the activity of the TCR can be controlled by light. Using this system allows us to inflict a series of ligand-TCR interactions and pauses for determined durations. We will use mathematical modelling to reveal how the kinetics of TCR triggering influences the outcome of T-cell activation.

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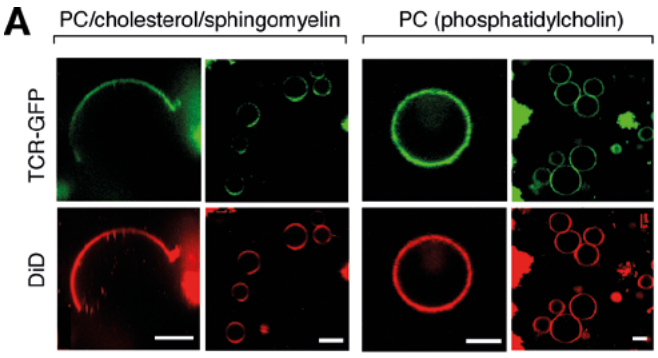
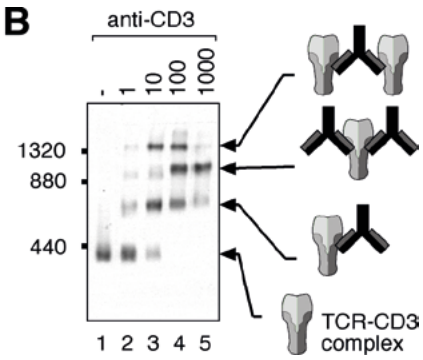


(A) We can determine the membrane microdomain localisation of proteins by reconstituting them in liposomes. The GFP-labeled TCR was reconstituted in LUVs composed of PC or PC/cholesterol/sphingomyelin. GUVs were generated from these proteoliposomes, and confocal images were taken. The ld domain was identified by DiD labeling. Scale bars, 5 µm.



(B) We can determine the stoichiometry of protein complexes by BN-PAGE using the NAMOS assay. Before loading digitonin-solubilized murine TCRs on BN gels, the indicated amounts (in nanograms) of the anti-CD3ε antibody 145-2C11 was added. After separation, immunoblotting was performed with an anti-CD3ζ serum to visualize the TCRs. Details on the method can be found in Swamy *et al.* J Immunol Methods, 2007. Images: Wolfgang Schamel

T cell activation in clinically relevant settings

Using mouse models as well as samples from immunodeficient patients, in close collaboration with groups of the Centre for Chronic Immunodeficiency (CCI), we study T- and B-cell activation in the context of immunologically relevant diseases. Lastly, we study a novel protein called Kidins220 that we have discovered to be bound to the TCR and BCR[6].

METHODS

Reconstitution of transmembrane proteins in Large Unilamellar Vesicles (LUVs)

Proteins reconstituted in LUVs can be observed in a defined lipid environment, without the presence of other proteins, thus this is an excellent method to study the effect of lipids on protein clustering. We have so far established the protocol for the TCR, the BCR, and the transferrin receptor. Due to the complexity of the molecules we wished to study, bacterial protein production was not possible, instead we used Streptavidin-Binding Peptide (SBP)-linked constructs (where the tag was engineered onto the chain that was the last in the line of assembly or the first to dissociate at degradation), and purified the complexes from T- or B-cell lines. In order not to destroy the complex, the lysis conditions had to be carefully regulated. The detailed description of the method is to be found here[2]. For the incorporation, we used pre-formed LUVs. For the reconstitution, low-dose Triton X-100 was found to be the most suitable detergent.

Lipid pull-down assay

Specific interactions between proteins and the polar head groups of membrane lipids have previously been described, but the interactions between the proteins and the lipid acyl chains are less understood. We therefore developed a method that combines a fatty acid or cholesterol pull-down assay with biochemical analysis (Western blot or mass spectrometry), in order to identify preferential interactions between transmembrane proteins, and various acyl chains or cholesterol. To this end, different acyl chains (short, long, saturated, and unsaturated) and cholesterol-coupled sepharose beads were synthesized and used in a pull down assay[7].

Lipid proximity analysis using radioactively labelled photoactivatable lipids

The use of radioactively labelled photoactivatable lipids enables us to investigate protein-lipid interactions *in vivo*. These lipid analogues, when activated by UV light, cross-link to molecules in their close proximity. We focused our studies on the interactions of TCR and cholesterol in the plasma membrane of T lymphocytes, therefore we cultivated the T-cell lines in the presence of [3α-^{3}H]6-Azi-5α-cholestan-3β-ol (photocholesterol) (from Prof. Dr. Christoph Thiele, LIMES Institute, Bonn/Germany). After TCR immunoprecipitation, SDS-PAGE was performed and analysed by both Western blot and autoradiography, in order to detect specific interactions. The method is described in detail here[2].

Blue Native (BN)-PAGE

BN-PAGE allows the separation and identification of native proteins and multiprotein complexes (MPCs). This method is best suited for the study of constitutive, abundant MPCs, such as multisubunit receptors or transcription factors. BN-PAGE has a higher resolution than gel filtration or sucrose density ultracentrifugation, and in contrast to immunoprecipitation or two-hybrid approaches, it allows the determination of the size, the relative abundance, and the subunit composition of the MPCs. While the one-dimensional BN-PAGE shows, how many different complexes exist that share a common subunit,

the two-dimensional BN- and SDS-PAGE gives more detailed information on the subunit composition. Detailed protocols of both are found in Swamy *et al.* 2006. *Sci STKE* 2006, pl4.

Furthermore, we developed two BN-PAGE-based methods, in order to answer specific questions regarding MPCs. An electrophoretic immunoshift assay, using monoclonal subunit-specific antibodies (NAMOS-assay) was developed for the determination of the stoichiometry of MPCs, while using a second method based on gel-retardation, the interactions of different biomolecules with protein complexes may be studied.

Optogenetic switch to control T-cell activation

In order to study the kinetics of T-cell activation, we collaborated with the labs of Prof. Dr. Matias Zurbriggen and Prof. Dr. Wilfried Weber. We constructed and expressed a human TCR, whose activity can be controlled by light, in T cells. Our laboratory is equipped with one-wavelength-illuminated cell culture rooms and computer-controlled LED boxes, where through illumination with 660 and 740 nm light, we can precisely determine the duration of the ligand-TCR interaction and the pauses in between.

Robot

We have a robotic platform including all liquid handling, a 37°C, CO_2 cell culture incubator with 15 “light boxes” to illuminate the cells with three different wavelength and a flow cytometer to generate standardized, accurate, and quantitative data sets. With this fully automated pipeline one can stimulate cells and perform cellular assays (such as intra- and extracellular FACS stainings) or biochemical assays (such as immunoprecipitations). In case of interest please contact us.

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Synthetic Cellular Microenvironments/Biofunctional Macromolecular Chemistry

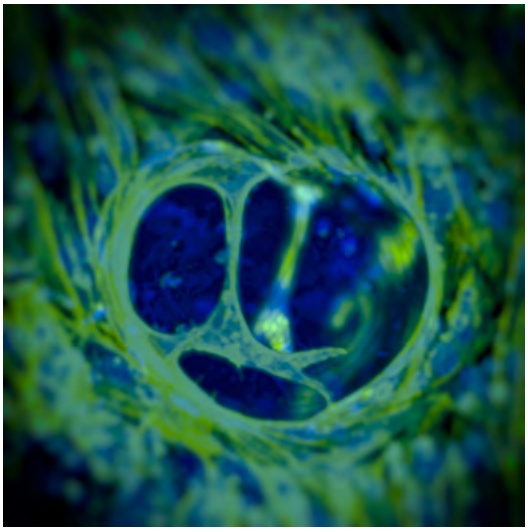
HEAD Prof. Dr. Prasad Shastri

Our research is focused on deciphering the function of biophysical parameters in trafficking of information into cells, cellular function, cellular organization, and tissue morphogenesis. We address scientific questions at the confluence of materials science, cell and molecular biology, and translational research. Using well defined material-based systems as cellular probes or synthetic mimics of cellular function, cellular structures, or their microenvironment, we are unravelling the molecular players in various cellular processes. This research effort borrows from several disciplines and leverages many techniques and methods.

PROJECTS

Synthetic extracellular matrices (ECM)

By implementing a knowledge-gain cycle that combines molecular modelling with polymer chemistry and secondary structure analysis, we have established new structure-property-function relationships in natural polysaccharide-derived hydrogels. This platform enables the investigation of how ECM structure and mechanics influence cellular organization. Using these mechanically defined hydrogels we have unravelled the critical role of ECM stiffness in the apical-basal polarization of endothelial cells and their organization into vascular structures.



3-D organization and branching of human endothelial cells into vascular trees in carboxylated agarose gels
© Aurelien Forget, Prasad Shastri

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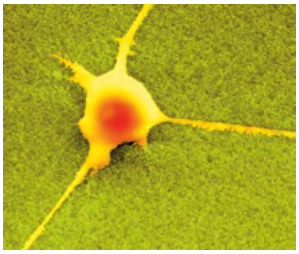
Role of Biophysical cues in central nervous system (CNS) physiology and cellular homeostasis

Macromolecules such as sulfated proteoglycans are abundant in the physiological environment and they have a role in growth factor binding and maintenance of tissue osmolality. The abundance of proteoglycans in the CNS prompted us to explore the role of nano-scale stochastic roughness, which is a direct manifestation of macromolecular structure, in the interaction of cells within the CNS and their function. We have discovered that interaction of neurons with astrocytes has a biophysical basis and that nanoroughness of the astrocytes directly influences neuronal health and function. Furthermore, the interaction between neurons and astrocytes involves Piezo-1, a stretch activated cation channel and its expression is critical for neuronal health and its dysregulation a hallmark of Alzheimer's.

METHOD

Atomic force microscopy (AFM)

AFM is a technique to characterize the surfaces of synthetic and biological materials. In AFM the surface of interest is probed using a nanometer sized tip either by dragging the tip along the surface or by repeatedly tapping the tip on the surface at a particular frequency. By varying the scanning parameters and the nature of the tip (stiff versus soft) surface topography and mechanical properties can be mapped. Furthermore, by chemically modifying the scanning tip with proteins and ligands, biomolecular interactions (ligand-receptor interactions) can be characterized. We use AFM routinely to visualize single macromolecules and image the surface properties (stiffness, topography) of live cells.



Scanning electron micrograph showing a neuron on a nanorough surface making intimate contact with the surface. Surfaces has been given a false colour for visualization. Source: Nils Blumenthal and Prasad Shastri

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Data Analysis and Modelling of Dynamic Processes in the Life Science

HEAD Prof. Dr. Jens Timmer

PROJECTS

Model-aided construction of light inducible gene expression systems
In contrast to chemical controllable systems, light controllable synthetic systems offer a high potential to interfere biological processes with unprecedented spatiotemporal resolution. Therefore, we support the construction of different light inducible gene switches in the group of Prof. Dr. Wilfried Weber by mathematical modelling.

PhyB/PIF6 system
With the photoreceptor phytochrome B (PhyB) and the phytochrome-interacting factor 6 (PIF6) of *Arabidopsis thaliana* combined with the chromophore phycocyanobilin (PCB) a bi-stable toggle switch to control gene expression with red and far-red light was developed. This system is described with a calibrated mathematical model based on ordinary differential equations (ODE)[1]. This model can be used in further applications, e.g. when this toggle switch is implemented in larger systems. The model was also used to calibrate a PCB synthesis pathway, which was implemented synthetically in mammalian cells.

UVR8/COP1 system
When illuminated with 311 nm UVB light, the UVR8 receptor can bind to the WD40 domain of COP1. This effect was used to construct a UVB light inducible gene expression system. We developed an ODE based model describing this system and calibrated the model with experimental data (Fig. a and b). With the model, it is possible to predict the target gene promoter activity (Fig. c) and to find optimal illumination conditions to obtain high target gene induction while minimizing cytotoxic side effects of the UVB light (Fig. d). The analysis showed that with pulsed light (5 minutes ON and 25 minutes OFF) it is possible to achieve very high target gene expression with cell viability near 100 percent[2].

Combination of PhyB/PIF6, UVR8/COP1 with the blue light sensing LOV system:
With the LOV domain of vivid (VVD), it is possible to construct a blue light inducible gene expression system. By implementing all thee receptors into single mammalian cells, one can control different genes by different light colours. Since the absorption spectra of the three light receptors PhyB, UVR8, and LOV are overlapping, the resulting system is not orthogonal, e.g. the blue light system can be activated by UVB light. Our mathematical analysis of the system showed that changing the light sensitivity of the LOV module is leading to full orthogonality of the three receptors and therefore to the first orthogonal optogenetic triple-gene control system in mammalian cells[3].

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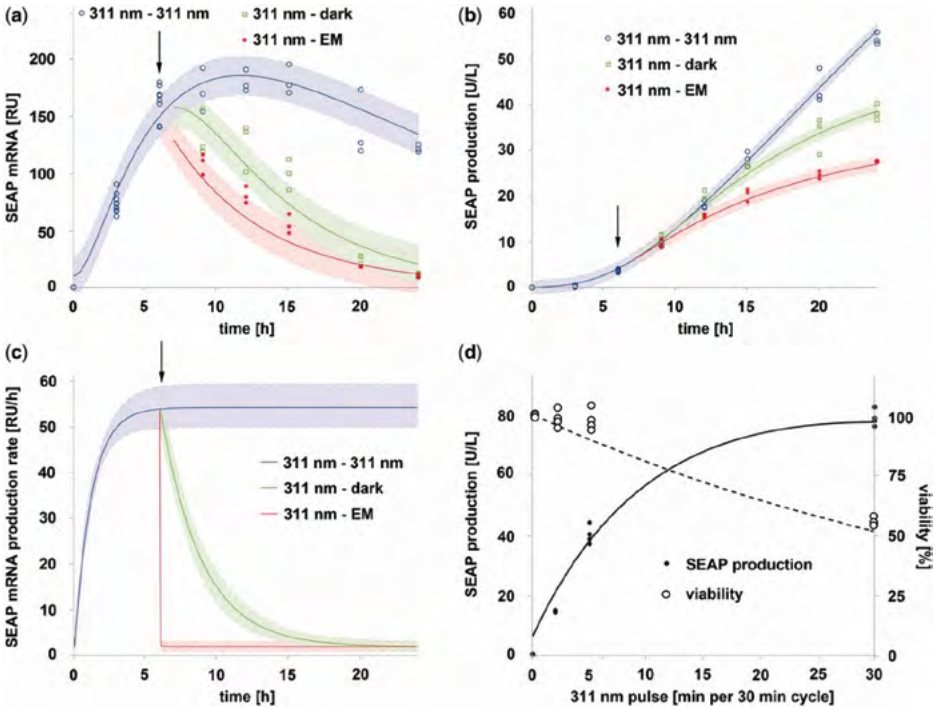

Model-based quantitative characterization of UVB-inducible gene expression [2].

(a and b) Light-inducible expression kinetics. CHO-K1 cells were engineered for UVB-inducible SEAP expression. After 24 hours, the medium was exchanged, and the cells were illuminated for 6 hours at 311 nm and were then either kept under 311 nm, moved to darkness or were supplemented with erythromycin (arrow). The SEAP mRNA (a) and protein (b) levels were determined at the indicated points in time. The curves represent the model fit to the data, and the shaded error bands are estimated by a simple error model with a constant Gaussian error. (c) Model-based analysis of expression kinetics. The SEAP mRNA production rate per promoter is shown for cells subjected to the stimuli used in (a and b). The shaded bands indicate the 95% prediction confidence interval. (d) Model prediction for SEAP production and cell viability under pulsed light (indicated in min light per 30 min cycle). The model predictions for SEAP production and cell viability are represented by the lines, whereas experimental validation data are shown as circles.
Figure: Konrad Müller and Raphael Engesser

METHODS

Mathematical modelling of modular designed synthetic systems in mammalian cells

The main focus of our group in BIOSS is to translate mathematical methods used in systems biology to the field of synthetic biology. On the one hand, this is done by model-guided construction of new modular designed synthetic biological systems with a desired behaviour, on the other hand by optimizing given synthetic systems by applying mathematical methods, e.g. *sensitivity analysis* to find good interaction points to interfere the system or *optimal experimental design* to find the best experimental conditions to run the system. The modular structure of synthetic networks simplifies the construction of mathematical models based on ordinary differential equations (ODE). Each synthetic motif and module has a defined function, which is described by a small ODE model. By wiring different modules to a bigger network, new intricate behaviour occurs. With the mathematical models of the smaller building blocks it is possible to simulate the behaviour of the larger network *in silico*, e.g. to find the optimal network structure to obtain a desired systems behaviour. By estimating the unknown model parameters from experimental data, one can make quantitative predictions. With this approach it is also possible to find the right dosing of the system modules for a given network structure, to obtain a robust and sustainable output of the synthetic system[4].

Estimating unknown model parameters from experimental data and handling uncertainties

Mathematical models of biological systems usually contain many unknown dynamical parameter like chemical reaction rates, protein degradation rates, or mRNA production rates. One major topic in systems biology and in modelling of synthetic systems is to estimate these dynamical parameters from experimental data. Biological systems typically are only partially observed, the measured data usually contains a high uncertainty in terms of measurement error but also biological variability and the considered models are highly nonlinear. This renders the parameter estimation very challenging. It is often difficult (i) to find the parameter set, which can describe the data the best, (ii) to draw a statistical correct interpretation of the resulting parameter set in terms of uncertainty. For the first point, our group develops new strategies to optimize such partly observed nonlinear ODE systems, based on deterministic *optimization of the likelihood* combined with sampling methods to identify local optima. For the second point, we use the *method of profile likelihood* to determine the uncertainty of the model parameters. This uncertainty can have different sources. On the one hand, the nonlinear model translates the uncertainty of the experimental data to an uncertainty of the estimated parameters. On the other hand, an insufficient amount of measurement data, e.g. an unobserved protein, is leading to non-identifiable parameters, e.g. the production rate of this protein. To capture these effects we developed a method to analyse the *parameter identifiabilities* using the profile likelihood[5].

Model predictions and experimental design

Mathematical models of biological systems are often used to make predictions, e.g. of the effect of an inhibitor. Uncertainties in the estimated parameters are leading to uncertainties in the predictions. To quantitatively determine these uncertainties in terms of prediction confidence intervals, we generalized the profile likelihood to the *prediction profile likelihood*[6]. With this method, it is also possible to perform an *experimental design* to find good experiments to obtain maximum information and reduce the uncertainties in the parameters. This is done by simulating the possible experiments *in silico* and evaluate the prediction profiles of the candidate experiments. The measurements with the highest predictions uncertainty are giving the most information.

Model selection

In systems biology the model structure is often unknown and one goal is to identify a model, which can describe the data. Usually there are many candidate models and a given set of measurement data and the task is to select one of the candidate models, which is in a statistical sense the best model. For this, we apply different model selection methods, like the AIC information criterion or likelihood ratio tests. Another common task is to find minimal models, which still can explain the data and capture all relevant biological effects. To solve this task we are adapting L1 penalization methods and combine them with the profile likelihood method.

Input estimation

Often an unknown or only partly measured external stimulus, like a hormone or a drug, is driving the biological system. Mathematically spoken, this external system input is a time dependent parameter. To simulate the model, the input has to be known for all time points. In general, the input is only measured at discrete points in time. Therefore, one has to estimate the value of the input between the measurements. For this purpose, we developed different mathematical methods to estimate the input simultaneously with the unknown dynamical parameters. One method is based on an approximation of the input function by cubic smoothing splines. A more general method, which can also be used to estimate very sparsely measured input functions, uses the calculus of variations motivated from the Lagrangian mechanics to estimate the input function[7].

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Molecular Imaging

HEAD Jun.-Prof. Dr. Maximilian Ulbrich

PROJECTS

Composition of the AMPA receptor

In our laboratory, we are interested in deciphering the assembly of the AMPA receptor, which is the main excitatory neurotransmitter receptor in the central nervous system. Due to its diverse functions in the brain, the AMPA receptor has a variety of different core and regulatory subunits which are known to bind to each other. However, the exact stoichiometry and the assembly rules are mainly unknown. With a unique single molecule imaging approach, we count the number of each subunit type in individual receptor complexes, and can even decipher complex assembly rules, e.g. cooperative binding and competition of regulatory subunits for binding sites at the receptor core (Hastie *et al.* 2013).

EGF receptor signalling

The epidermal growth factor receptor (EGFR) promotes proliferation and motility in epithelial cells. Therefore, it has an important role not only in development and growth, but also in cancer where dysregulation of the receptor leads to invasion of cells into other tissues. Many approved cancer drugs directly target the EGFR or other members of the ErbB family (ErbB2, ErbB3, ErbB4). A large body of knowledge has been generated through classical methods, but dynamic processes during receptor activation at the plasma membrane remain hidden to most techniques. With single molecule imaging, we are able to directly observe the movements of the receptors at the cell surface and characterize the first steps of activation.

Synthetic membrane signalling systems

To better understand signalling of membrane proteins on a physico-chemical level, we rebuild membrane signalling systems from modular components, e.g. transmembrane domains, inducible dimerization domains, and fluorescent proteins for readout. By single molecule imaging we can directly observe association and dissociation events of the synthetic signalling units and develop a model for describing the dynamics of lateral interactions of membrane associated proteins. Our goal is to mimic natural signalling proteins, e.g. surface receptors, and to rebuild functional pathways that are orthogonal to cellular mechanisms.

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METHODS

Single molecule imaging

Our group is specialized on the single molecule imaging technique. With the use of very sensitive equipment and custom designed microscope setups, we are able to directly image fluorescently labelled molecules. To achieve a very high signal to noise ratio, we use Total Internal Reflection Fluorescence (TIRF) that restricts the illuminating light to the surface of the substrate (e.g. the coverslip). This technique allows us to image single membrane proteins in living cells or protein complexes with fluorescent dye tags attached.

Subunit composition of membrane proteins

With single molecule imaging in several colours, it is possible to determine the assembly of protein complexes (Ulbrich and Isacoff 2007). Because every fluorescent dye molecule photobleaches in a discrete step, it is also possible to determine the multiplicity of each subunit in individual complexes. This enables us to decipher the stoichiometry of subunits in larger protein assemblies, and even to determine whether a certain assembly pattern is preferred over others in the context of a living cell (Bartoi *et al.* 2014).

Diffusion and cluster formation of membrane proteins

The dynamic interactions of proteins at the cell surface are key to understanding signalling events on a biophysical level. With single molecule imaging and related imaging techniques, our lab studies the dynamics of activation for the EGF receptor and other receptor tyrosine kinases during signalling events. We can directly observe and quantify the diffusion of the proteins in the plasma membrane and the cluster formation upon stimulation with a ligand.

Xenopus laevis oocytes

The oocytes of the African clawed frog *Xenopus laevis* are an ideal expression system for membrane proteins to perform optical and electrophysiological experiments. Instead of transfecting DNA, expression in *Xenopus* oocytes is achieved via injection of RNA. Since this system bypasses the difficult to control process of transcription, it allows simultaneous expression of multiple proteins at well-defined levels in the same cell, which is an ideal situation for protein interaction studies.

The oocytes of the African clawed frog *Xenopus laevis* are an ideal expression system for membrane proteins to perform optical and electrophysiological experiments. Photography by: Th.Totz-photography

Optical setups

No commercial equipment exists for single molecule imaging. Therefore, the members of our lab are assembling the optical setups by themselves. This allows us to optimize the method to the specific application and go beyond what is possible with commercial setups that are a compromise between many different requirements. With a modular setup it is also possible to react to current developments in technical or biological respect, e.g. new cameras or new fluorescent proteins.

Nanobodies

Nanobodies are fragments derived from camelid antibodies, but are only about 10 percent of the size of conventional antibodies, while still retaining their high affinity to their antigen. An additional advantage is that they can be expressed in any cell type, even in bacteria. We use nanobodies for fluorescent labelling of target proteins with high affinity and for generating scaffolds for protein interactions. We use nanobodies with different specificities, but the nanobody against GFP is the most commonly used and performs excellent in our hands.

CRISPR/Cas9

We use this novel technique for editing the genome of cell lines that we use for our microscopy experiments. Since the EGF receptor uses cellular signalling pathways that are present in most cell lines, we modify endogenous pathway components to understand the impact on the signalling of the EGF receptor.

Viral gene delivery

The delivery of genes by viral vectors has the advantage that stable cell lines can be generated in much less time than by transfections of plasmid DNA. We use lentiviral expression vectors but also take advantage of a viral gene delivery system based on the murine leukemia virus. This system has the advantage that the viral particles cannot infect human cells because of the high specificity for mouse and therefore can be used under biosafety level S1. To use it with cell lines from non-murine origin, the cell line can be transiently transfected with the receptor for the murine virus to confer susceptibility to infection.

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Kidney Tubule Formation and Regeneration

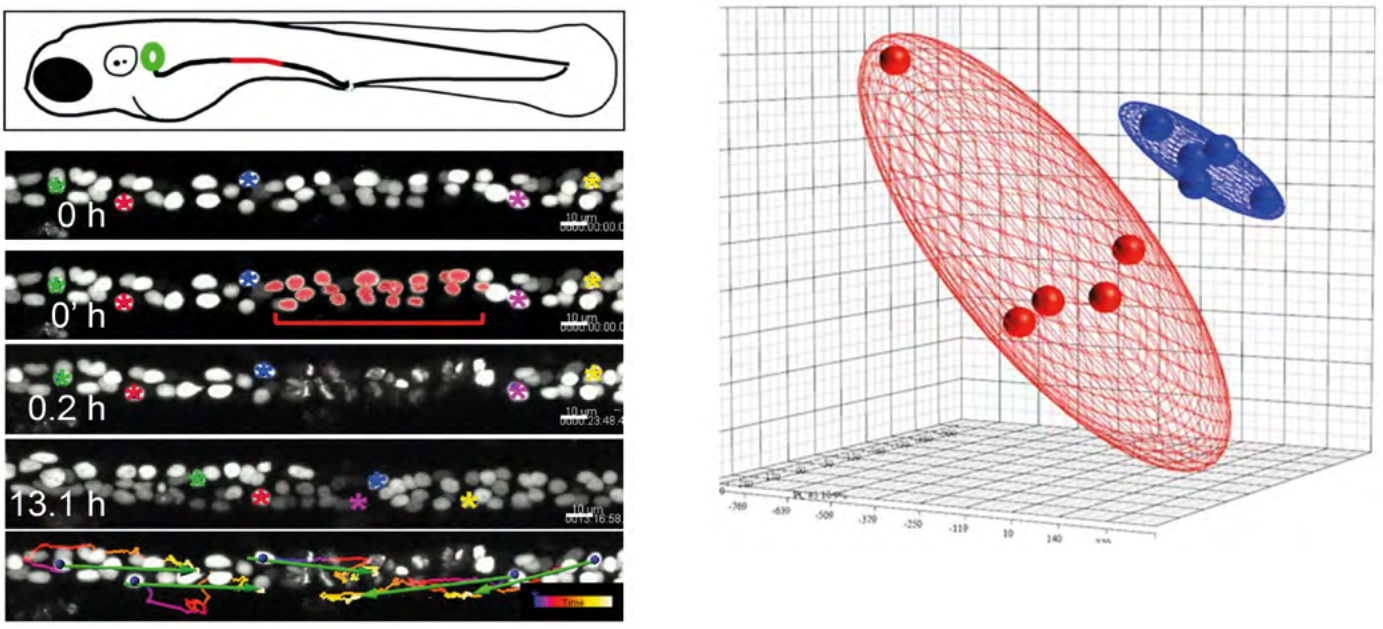
HEAD Prof. Dr. Gerd Walz

PROJECTS

What drives cells to collectively establish a filter (glomerulus) and a tubule that ultimately form the filtering unit (nephron) of a mammalian kidney? We decided to analyse the developmental programmes that instruct tubular epithelial cells to generate the elongated structure that is characteristic for a mammalian nephron. The precisely defined final geometry originates from reciprocal interactions between the ureteric bud and undifferentiated mesenchyme. The subsequent nephron formation is characterized by episodes of massive cell proliferation, which are intersected by coordinated cell migration that progressively extends the renal tubule to its final length. The underlying programmes controlling the collective cell migration are difficulty to study in the mammalian kidney *in vivo*. Since *Xenopus* embryos are transparent, time-lapse confocal imaging can be utilized to approach this question and determine the fundamental programmes involved in kidney tubule formation in this model organism. We found that renal tubular cells are highly dynamic, and continuously form rosettes where five to seven cells join at a shared vortex. After a slight rotation, these rosettes dissolve again to form new rosettes with other neighbouring cells. This cell migration pattern represents an extremely effective way to extend globular cell aggregates into long extended structures. Perhaps not surprisingly, a similar programme is utilized already by the fruit fly *(Drosophila)* for germ band extension, suggesting that tissue elongation by rosette formation represents a highly conserved evolutionary programme. Furthermore, analysis of mouse kidney sections confirmed the presence of rosettes, providing clear evidence that this programme is also utilized in the mammalian kidney. Interestingly, cell cycle inhibitors that prevent cell proliferation only partially interfere with tubule elongation, suggesting that cell proliferation does not contribute significantly to the nephron extension during early pronephros development in *Xenopus* embryos.

Kidney cysts form when cellular programmes fail to establish a nephron with a genetically defined geometric structure. Cilia, present on the apical membrane of tubular epithelial cells, are required for nephron development and a normal tubular architecture; ciliary defects almost universally cause a cystic out pouching of the kidney tubules. Although the molecular connecting is not understood, cilia appear to influence the position of the mitotic spindle during cell proliferation, and intersect with the non-canonical Wnt signalling cascade required for normal rosette formation and coordinated cell migration. One readily accessible model system to study cilia is the Xenopus epidermis that contains single cells with multiple motile cilia. These cilia are polarized to generate a directional fluid flow along the longitudinal body axis of the embryo. We observed that nephrocystins, a gene family mutated in an autosomal recessive form of cystic kidney disease, interact with the planar polarity protein

bioss.science/pm/kidney_tubule_formation



Laser-mediated pronephric duct injuries are repaired by cell migration in the zebrafish embryo. The left panel depicts the cells (red) ablated by laser. Videomicroscopy of the injured region reveals that the gap is rapidly closed by neighbouring cells. The right panel depicts the microarray results of micro-dissected zebrafish pronephric ducts at one and two days post fertilization (dpf). Image: Research group Walz

Inturned and actin-nucleating factors to modify the local actin cytoskeleton. A precisely configured apical actin web, however, appears to be mandatory for normal ciliogenesis and tubulogenesis.

Since tissue development and regeneration share many features, the finding that the developing kidney tubule is predominantly shaped by cell migration prompted a central question: How important is cell migration during the repair process after kidney injury? Acute kidney injury associated with tubular necrosis frequently accompanies severe human disease, for example sepsis. Caused by hypoxia often in combination with toxins, tubular epithelial cells undergo necrosis and other forms of cell death (e.g. apoptosis). Our current knowledge of the repair process is based on static analyses of sequential tissue sections obtained several hours (or days) after the initiating event. This approach has led to the assumption that proliferation of neighbouring cells or perhaps nearby stem cells are essential components of the repair process. However, the technical limitations to visualize the repair process directly have obscured the early repair programmes. To address this question in a more stringent manner, we analysed the zebrafish pronephros repair process by time-lapse imaging after ablating tubular epithelial cells by laser. Following the repair process by high-resolution video-microscopy revealed that the injury overrides other developmental programmes, for example the posterior-to-anterior collective cell migration characteristic for this stage of development, and reversed the migratory path of cells immediately adjacent to the injury to close the laser-induced wound. Micro-dissection and transcriptional profiling of the zebrafish pronephros uncovered an up-regulation of distinct families of adhesion and tetra-membrane-spanning proteins that have previously been implicated in inflammation and tumour metastasis. Knockdown of these candidates slowed or inhibited the repair process underlining their potential involvement in tubular repair, and revealing a similarity between programmes that control migratory repair and metastasis. Using transcriptional profiles in combination with proteomics to determine the interactome of candidate proteins, we are currently establishing a network of transcription factors and adhesion molecules that control the repair process after tubular epithelial cell injury. These observations may lead to novel approaches to treat acute kidney injury and tubular necrosis, a challenge that has not been successfully addressed over the past couple of decades.

METHODS

High-resolution video-microscopy

We use high-resolution video-microscopy in living organisms such as *Xenopus* and zebrafish embryos to analyse developmental programmes *in vivo*. To capture complex cell movements, special software algorithms are developed by BIOSS (Dr. Olaf Ronneberger).

Micro-dissection and laser-capture of zebrafish pronephric ducts

To determine the special gene expression patterns in the zebrafish pronephros at different developmental stages, pronephric ducts were micro-dissected, and subjected to micro-array analysis. To determine the specific expression profiles in tubular epithelial cells immediately adjacent to laser-ablated cells, and thus involved in wound repair programmes, the laser-capture technique was adapted to visualize and isolate mRNA from fluorescently labelled pronephric ducts after laser-mediated injury.

The *Xenopus* epidermis as a model system to study motile cilia

Xenopus laevis can be readily manipulated by micro-injection of morpholino oligonucleotides (MOs). To avoid overall embryonal malformation, the MOs can be targeted to specific organs and tissues. High- and super-resolution confocal imaging can then be used to examine various aspects of ciliogenesis (e.g. basal body docking, polarization, actin cytoskeleton dynamics).

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Biochemistry and Functional Proteomics

HEAD Prof. Dr. Bettina Warscheid

PROJECTS

Regulation of the peroxisomal importomer

Import of matrix proteins into peroxisomes is facilitated by a large, membrane-bound translocation machine, the peroxisomal importomer. The translocation process is mainly governed by the peroxins Pex14p and Pex5p, which together form a highly dynamic pore. To date, information about the dynamic regulation of this central gateway to peroxisomes is lacking. We found that Pex14p and Pex5p are phosphorylated at multiple sites in their mutual binding regions. Several cytosolic kinases act differently on these proteins. Using yeast genetics and biochemical methods, we study the impact of Pex14p/Pex5p phosphorylation on pore formation and matrix protein import under different conditions.

Signalling in and out of the Z-disc

The myofibrillar Z-disc is an essential, structure-bearing component of muscle fibers. While originally considered as a passive unit, our group seeks to better understand its evolving role in myocyte signalling. Using large-scale phosphoproteomics, we revealed the Z-disc as the major site of protein phosphorylation in sarcomeres. We identified the large actin-cross-linking protein filamin C (FLNc) as signalling hub. Mutations in the gene coding for FLNc cause severe muscle diseases (filaminopathies). We found that kinase-mediated phosphorylation of FLNc significantly alters its dynamic behaviour in cells. We further use proximity proteomics approaches to study phosphorylation-dependent changes in the *in vivo* nano-environment of FLNc and perform kinase-substrate correlation profiling on a system-wide scale with the aim to advance our understanding of hypertrophic signalling cascades in myocytes.

The phosphatase-substrate network in B cells

Dysfunction of protein tyrosine phosphatases (PTPs) can lead to hyperactivity and auto-reactivity of B cells. Tyrosine phosphorylation, tightly controlled by PTPs, plays a fundamental role in the signal transduction of the B cell antigen-receptor complex and its co-stimulatory receptors. Together with the Reth group, we delineate the PTP-substrate network in B cells to obtain a better understanding of the molecular mechanisms underlying B cell autoimmunity. We quantitatively analyse changes in the phosphotyrosine proteome in WT *versus* PTP-deficient cells (CRISPR/Cas9) and make use of PTP trapping mutants to determine their *in vivo* substrates by quantitative mass spectrometry.

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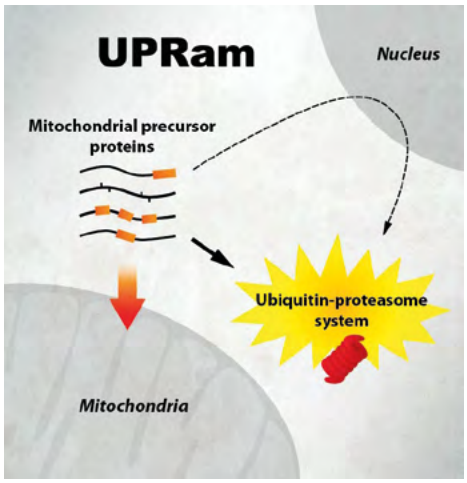
Redox signalling

Mitochondria are a major source of reactive oxygen species (ROS) in cells. Accumulation of intracellular ROS causes oxidative stress and this physiological condition as well as mitochondrial dysfunction have been associated with human diseases (e.g. cancer, Alzheimer's disease) and ageing. We established an advanced global redox proteomics approach to delineate the protein oxidation landscape of cells under basal and oxidative stress conditions as well as during ageing. We identified a set of mitochondrial proteins with elevated oxidation and/or expression levels which is the basis for further functional studies. Moreover, we recently discovered a new mechanism called UPRam (Unfolded Protein Response activated by mistargeting of proteins) that protects cells against the accumulation of mitochondrial precursor proteins due to a defect in the protein import system (Wrobel *et al.* 2015). We showed that the cell maintains its inner balance as it adjusts the mechanisms for the regulation of the cellular protein homeostasis to the state of its mitochondria. The cell activates the proteasome by a more efficient assembly and, thus, reacts directly to the amount of proteins that fail to be imported into the mitochondria.



Unfolded Protein Response activated by mistargeting of proteins (UPRam) consists of two pathways that are used by the cell to protect itself against a defective mitochondrial protein import. First, the cell inhibits translation to counteract further accumulation of mitochondrial precursor proteins. Second, the cell activates the proteasome which removes the already existing traffic jam on the mitochondrial protein import pathway. Graphics: Agata Trojanowsk

Our data may help to better understand mechanisms of age-related and neurodegenerative diseases in the future. These are often accompanied by mitochondrial dysfunctions and the impairment of proper protein homeostasis.

METHODS

High-resolution mass spectrometry

Rapid advancements in mass analysers as well as ionisation and dissociation methods of biomolecules elevated mass spectrometry to a key technology in biological and medical research. In our research, we use different types of high-resolution *orbitrap instruments* connected to ultra-high performance liquid chromatography *(UHPLC)* systems for effective peptide separation prior to highly sensitive electrospray ionisation tandem MS analysis. Furthermore, we employ *orthogonal protein and peptide separation* technologies including size exclusion, ion exchange (cation or anion) as well as low and high pH reversed-phase HPLC to obtain deep proteome coverage with up to 10,000 protein identifications. For *label-free* quantitative analysis and *protein correlation profiling* (Niemann *et al.* 2013), we perform feature analysis in MS1 scans using the MaxQuant or Skyline software package. Ultra-fast peptide sequencing is facilitated by collision-induced dissociation *(CID)* or higher-energy collisional dissociation *(HCD)* technologies. Applying advanced fragmentation methods including multi-stage activation *(MSA)* and electron transfer dissociation *(ETD)*, we are able to locate multiple post-translational modifications *(PTMs)* in proteins. For example, we identified the serine-rich hinge 2 region of FLNc as a hotspot of phosphorylation events mediated by several cytosolic kinases. In selected applications, we monitor specific targets by multiplexed single ion monitoring *(msx-SIM)* or develop *top-down* proteomics approaches.

SILAC technology

Over the last 10 years, *SILAC* (stable isotope labelling by amino acids in cell culture) has advanced to a prime tool in quantitative and functional proteomics research. The virtue of SILAC is its versatility, which led to the development of a large array of different SILAC approaches using various model organisms. The main feature of the SILAC technology is the potential to label the entire proteome with defined combinations of stable isotopes during an organism's growth. We use SILAC for the metabolic labelling of the yeast *Saccharomyces cerevisiae*, the protozoan *Trypanosoma brucei*, and various mammalian cell lines (HEK293, HeLa, C2C12, Ramos etc.). To overcome issues with the use of auxotrophic strains for complete SILAC labelling, we established a *native SILAC* approach. Furthermore, we employed *pulsed SILAC* methods to study proteome dynamics and to identify microRNA targets by the analysis of newly synthesized proteins using high resolution MS (Hünten *et al.* 2015). Following the *super-SILAC* concept, we also generated SILAC-labelled reference proteomes as

spike-in standards for quantitative proteome analysis across different developmental stages of cells in culture and entire organisms.

Chemical tagging methods

The synergy of stable isotope tagging and high-resolution MS provides the unique opportunity to accurately quantify proteins on a system-wide scale. Chemical tagging approaches are universally applicable (e.g. to tissue samples, primary cells) and, depending on the reactive group of the stable isotope tag used, different functional groups (amide, sulfhydryl, or carboxyl group) of peptides/proteins can selectively be targeted. To study different types of primary B cells, we employed the multiplex stable isotope *dimethyl labelling* method to modify the free N-termini of proteolytic peptides followed by multi-dimensional LC and quantitative MS. Alternatively, the *iTRAQ*™ (isobaric tags for relative and absolute quantification) or *TMT*™ (tandem mass tag) technology allow for sample multiplexing by reporter ion-based peptide quantification in MS/MS scans.

Signalling proteomics

Cellular signalling processes are mediated by reversible changes of PTMs which in turn may specifically alter the activity, stability, interactions, and/or subcellular location of a given protein. Among the different types of PTMs, *phosphorylation* plays a pivotal role for the modulation of signalling events in a cell. We established effective *phosphopeptide enrichment techniques* using TiO_2 beads and/or anti-pTyr antibodies that allow for the identification of thousands of phosphorylation sites by powerful LC/MS and bioinformatics methods. Aided by large-scale *quantitative phosphoproteomics*, we study complex kinase-substrate relationships in mammalian cells (Wiese *et al.* 2015; Schwarz *et al.* 2015). To untangle crosstalk between signalling cascades controlling cell fate decisions, we just started to exploit *optogenetic tools* (e.g. light-inducible kinases) for time-resolved phosphoproteomics studies. Furthermore, for the systematic study of *redox signalling* processes, we recently established a reversible thiol-trapping approach comprising cysteine-specific stable isotope labelling using *ICAT*™, quantitative high-resolution LC/MS, and advanced bioinformatics. Using this global *redox proteomics* approach, we were just able to simultaneously identify and quantify the reversible oxidation state of several thousand cysteine residues *in vivo*, allowing for the first time for the in-depth characterization of the yeast redoxome under basal and oxidative stress conditions.

Interaction proteomics

The fate and function of a cell depends on the smooth operation of a vast diversity of spatially and temporally regulated biological processes. At the molecular level, a single protein's function is often aided and modulated by various dynamic binding partners, which eventually results in the formation of larger *protein assemblies* and *interaction networks*. We established methods to effectively affinity-purify native membrane protein complexes under mild conditions and to subsequently delineate the entire membrane *interactome* by quantitative MS (Oeljeklaus *et al.* 2012; Mick *et al.* 2013). Alternatively, to define a protein's nano-environment *in vivo*, we exploit new *proximity proteomics* approaches. To this end, a promiscuous *Escherichia coli* biotin protein ligase mutant (BirA*) is fused to a protein of interest. Following expression of the fusion protein in cells, excess biotin is added to efficiently biotinylate binding partners and near neighbours. Subsequently, cells are lysed under denaturing conditions and *in vivo* biotinylated proteins are affinity-purified by Streptavidin for identification and classification by quantitative MS. We refined this proximity-dependent biotinylation method to learn more about the formation of dynamic protein assemblies of cytoskeletal proteins involved in sarcomere formation and to identify *in vivo* substrates of the protein tyrosine phosphatase PTP1B.

Cross-linking techniques

To identify protein-protein interfaces, we employ different protein cross-linking methods. Subsequent identification of inter- and intramolecular cross-links is facilitated by high-resolution LC/MS and computational approaches. For *chemical cross-linking*, we use the zero-length cross-linker ethyl diisopropyl carbodiimide (EDC) combined with sulfo-N-hydroxysuccinimide ester (sulfo-NHS) as well as stable isotope-labelled versions of the lysine-reactive cross-linkers disuccinimidyl suberate (DSS) and its sulfo analog bis(sulfosuccinimidyl) suberate (BS3), providing a maximum span of 24 Å (Nε-Nε distance). Alternatively, we perform *site-specific photo-cross-linking* facilitated by the genetic incorporation of the unnatural amino acid p-benzoyl-L-phenylalanine into the target protein. To establish spatial relationships of different subunits in multiprotein complexes, we affinity-purify native protein complexes from cells and conduct *in situ chemical cross-linking* followed by MS analysis. The acquired cross-linking data can be used as low-resolution distance restraints for modelling approaches within integrative *structural biology* approaches.

Nucleofection

This method is based on electroporation and enables the direct transfer of various biologically active molecules into the nucleus and the cytoplasm of mammalian cells. The use of the 4D-NucleofectorTM system (Lonza/Amaxa), the most advanced nucleofection platform currently available, allows for the efficient, *non-viral transfection* of *primary and non-dividing cells* as well as *hard-to-transfect cell lines* with high flexibility. Both high cell numbers (2 x 10^5 to 2 x 10^7 cells in 100 µl) and low cell numbers (2 x 10^4 to 1 x 10^6 in 20 µl) can readily be transfected. In addition, *adherent cells* can be transfected in 16-well nucleocuvettes (20 µl/well). We established this method to efficiently transfect mouse myoblasts. High expression of the target protein was observed in contracting myotubes seven days after induction of differentiation.

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Synthetic Biology

HEAD Prof. Dr. Wilfried Weber

PROJECTS

Optogenetic Switches

Our group works on the development of optogenetic switches to control cellular signalling processes in mammalian cells. In particular, we use plant-derived photoreceptors to control gene expression in response to red, blue, and UV light. By designing light-responsive split transcription factors based on *Arabidopsis* phytochrome B, *Avena sativa* LOV2 domains or *Arabidopsis* UVR8 we achieved mutually independent control of three transcription factors within a single mammalian cell by the selective illumination with light of the corresponding wavelength. In collaboration with other colleagues we work on the development of light-inducible kinases such as C-Raf (apl. Prof. Dr. Gerald Radziwill).

Small molecule-responsive hydrogels for the remote-controlled, inducible release of biomolecules in mice

We develop and apply stimulus-sensing hydrogels by crosslinking polyethylene glycol polymers by antibody-antigen or drug-target interactions. These hydrogels can be dissolved by the addition of small molecules (antigens, drugs) in a dose-dependent manner to release previously embedded cargo. We have synthesized such hydrogels with embedded vaccine cargo (against human papilloma virus or hepatitis B virus) and demonstrated that the implantation of such a vaccine depot in mice can be dissolved by the oral addition of small molecules thus releasing bioactive vaccine to boost the immune response. We are currently further developing such depots in the frame of a single-injection vaccination strategy.

Light-responsive hydrogels as remote-controlled extracellular matrix

By functionally coupling bacterial photoreceptors to polyethylene polymers, we develop hydrogels whose viscoelastic properties can gradually, locally, and reversibly be adjusted by multichromatic illumination. By incorporating integrin attachment regions into the hydrogel, we enable cells to grow on and in the matrix. We demonstrated that the light-responsive modulation of the matrix' properties can be used to control cell migration as well as mechano-signalling. This matrix will further be applied to analyse how signalling processes respond to dynamically changing mechanical environments.

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METHODS

Inducible gene switches

In addition to the optogenetic gene switches described above, we developed and apply a large variety of gene switches compatible with mammalian cells responsive to small molecules such as antibiotics (macrolides), amino acids, quorum-sensing messengers, auxin etc. Most of these switches have been shown to be functional in mice.

Analytical (metabolite) assays

By developing ELISA-like assays based on bacteria repressor proteins, we developed analytical tests for the quantification of metabolites, antibiotics, or other compounds in ELISA plates, in microfluidic devices, or electrochemical chips. These assays allow the simple quantification of selected small molecules in biological samples without the need for expensive lab equipment.

Production, characterization, and modification of proteins

We routinely use bacterial and mammalian systems for the production of proteins, apply different purification strategies, and have a large repertoire of technologies for specifically coupling small molecules or polymers to the proteins.

Illumination of biological samples

In collaboration with the electronics workshop of the Faculty of Biology, we have developed illumination devices for biological systems such as mammalian cells. The illumination devices can be operated in cell culture incubators and can easily be programmed via a universal serial bus (USB) connection to a computer to exhibit user-defined illumination kinetics and intensities of up to four different wavelengths.

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Molecular Genetics of Prokaryotes

Photograph: Ekaterina Kuchmina

HEAD Prof. Dr. Annegret Wilde

PROJECTS

Photoreceptors and regulation of phototaxis

Cyanobacterial phytochromes belong to a diverse family of light receptors controlling different biological cell functions including phototaxis. In addition to canonical *bona fide* phytochromes of the well characterized plant-type clade, cyanobacteria also harbour phytochromes that absorb green, violet, or blue light. We have analysed spectroscopic characteristics of light-absorbing domains. We found that the cyanobacterial phytochrome Cph2 is a four-colour light sensor which regulates phototaxis and other cellular functions by controlling intracellular c-di-GMP levels. We are combining genetic, biochemical, and structural analyses to unravel the mechanism how different light signals are processed and transmitted by one photosensor. This information will be correlated to the biological output, namely alterations in phototactic motility and c-di-GMP level. In addition, we aim to use cyanobacterial photoreceptors for optogenetic applications. In a closely related project, we want to elucidate the mechanism how cells detect the direction of light and how they move towards a light source.

Systems biology of cyanobacterial biofuel production

Cyanobacteria have the potential to become one of the future sources of biofuel. They can be used as effective producers of biomass, hydrogen, and ethanol because they have the distinct advantage of being able to use carbon dioxide as their primary carbon and sun light as the sole energy source. Our approach is to combine photosynthesis with the synthesis of ethanol in a cyanobacterial cell. Together with our academic and industrial partners, we want to establish a systemic understanding of a photosynthetic prokaryote by experimental analysis of relevant metabolic pathways and its regulation combined with data-based mathematical modelling. The understanding of cyanobacterial signalling systems will be a milestone on the way of developing industrial relevant expression strains for biofuels.

Posttranscriptional gene regulation in cyanobacteria

Recent data from pro- and eukaryotic organisms show the extremely high potential of non-coding RNAs (ncRNAs) as sequence-specific regulators of gene expression, thereby mediating a plethora of cellular responses to changing environmental conditions. Systematic searches for ncRNAs are still lacking for most bacteria outside the Enterobacteria mostly because genes encoding ncRNAs are not annotated during standard genome analysis procedures. In this project, we intend to unravel the function of RNA regulators as molecular switches to respond to environmental changes. We found several ncRNAs to be involved in regulation of photosynthetic functions in cyanobacteria. Further, we aim to analyse the function of RNA degrading enzymes in cyanobacteria using the iCLIP method.

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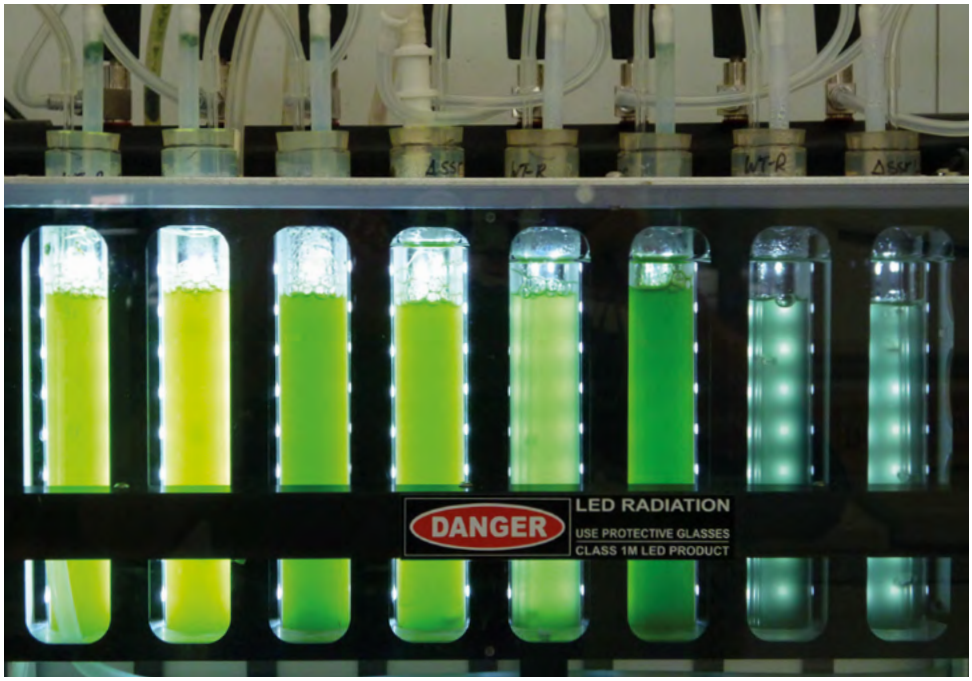
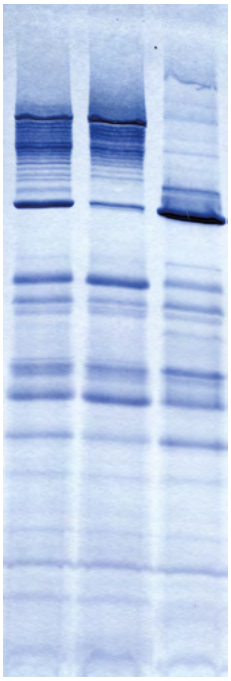


Small-scale photobioreactors for growing algae cultures under different light conditions. Photograph: Thomas Wallner



Blue-Native Polyacrylamide gel electrophoresis of cyanobacterial membrane complexes.
Image: Ulf Dühring

METHODS

Quantitative single-cell tracking under controllable light conditions

This microscopic technique allows the simultaneous tracking of multiple single bacterial cells relative to one or more adjustable LED light sources. We employ this system to evaluate the response of single cells (wild type and photoreceptor mutant cells) to differences in wavelength and orientation of a light source.

iCLIP method

Individual resolution crosslinking and immunoprecipitation in combination with high-throughput sequencing has been performed for RNA-binding proteins in bacteria. Using this method, the amino acids crosslinked to the RNA remain attached. This is crucial for precise monitoring of protein-RNA binding sites at nucleotide resolution after sequencing. Introduction of random barcodes allows quantification of the crosslinked RNA (Rossbach *et al.* 2014).

Absorption spectroscopy

We are using a spectrophotometer equipped with an integrating sphere to measure highly scattering pigment samples. The function of an integrating sphere is to spatially integrate radiant flux in scatter transmission.

Blue-Native PAGE

Blue native PAGE (BN-PAGE) is used for separation of membrane proteins and complexes in the range of 10 kDa to 10 MDa (Wittig *et al.* 2006). This technique allows isolation of membrane protein complexes from cell lysates to determine native masses and oligomeric state of protein complexes. In combination with Tris-Tricine PAGE in second dimension BN-PAGE, this is a powerful method to determine assembly, stoichiometry, and interactions of subunits in multi-protein complexes (Dühring *et al.* 2007).

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Tumour and Transplant Immunology

HEAD Prof. Dr. Robert Zeiser

PROJECTS

Positron emission tomography imaging for the detection of inflammatory neovascularization

Graft-versus-host-disease (GvHD) was for a long time considered to be a predominantly T cell mediated disease, which imposes a major problem for targeted therapy because T cells are also responsible for the majority of beneficial effects of allo-HCT, namely graft-versus-leukemia (GvL) and graft-versus-infection (GvI) effects. Our work has recently challenged this concept showing that inflammatory intestinal neovascularization[1] also contributes to the disease. By using Positron emission tomography imaging (PET) we could identify αvβ3 integrin expression within areas of intestinal neovascularization[1]. To interfere with neovascularization we targeted αv integrin-expressing endothelial cells by an integrin inhibitor, which blocked their accumulation in the intestinal tract and reduced GvHD severity[1].

Bioluminescence imaging for detection of myeloid cells and magnetic resonance imaging for bacteria tracking

In order to track the expansion of donor derived T cells during GvHD in the mouse, we have employed luciferase transgenic donor animals that express luciferase under the control of the β-actin promoter. Based on duplication of the luciferase reporter gene upon cell division the signal increases in quickly dividing cells and allows for efficient tracking *in vivo*. Conversely, to study slowly dividing myeloid cells, a novel method had to be established that allowed us to detect cells containing active Myeloperoxidase (MPO) based on the emission of light when the substrate luminol is metabolized by active MPO[2]. With this method, we have recently described myeloid cell infiltration into the intestines during early GvHD. Additional magnetic resonance imaging (MRI) of the intestines identified transmigration of superparamagnetic iron oxide (SPIO) labelled *E. coli* bacteria into the peri-intestinal tissue following total body irradiation (TBI). The MRI and MPO-imaging results were complemented by histological studies to identify and localize gram+ bacteria and polymorph nuclear neutrophils. Strong neutrophil infiltration was also found in GvHD lesions of patients with severe GvHD.

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METHODS

MRI

Comparable to our previous studies[3], we have established a procedure to label bacteria (wildtype *E. coli* serotype O157:H7) with iron oxide particles (SPIO, Ferucarbotran, SHU 555 A, Resovist®, Schering AG, Berlin, Germany). A dose of $1x10_7$ SPIO+ E. coli is given by rectal application to mice which have previously received Total body irradiation which is considered to damage the intestinal barrier. The bacteria transferred were shown to migrate in the peri-intestinal tissue by MRI. Additionally we found 16S RNA by qPCR indcating the presence of bacteria in the mesenteric lymph nodes. The SPIO solution was Ferucarbotran (SHU 555 A, Resovist®, Schering AG, Berlin, Germany), a clinically established contrast agent for MRI. It consists of SPIO nanoparticles (average diameter, 65 nm, and core size, 3-5 nm) coated with carboxydextran. One millilitre solution contains 540 mg Ferucarbotran, corresponding to 0.5 mmol (28mg) iron. SPIO-based contrast agents mainly act on R2 (1/T2) and R2 * (1/ T2*) relaxation rates. The use of SPIO nanoparticles allows higher resolution of the anatomical context in which the bacteria reside and was therefore preferred to FN based labelling.

Fluorescence- and *in vivo* bioluminescence imaging (BLI)

We have previously generated luciferase transgenic mice from which T cells can be isolated and traced *in vivo* by using a CCD BLI camera after injection of the substrate luciferin [D-Luciferin 1-(4,5-dimethoxy-2-nitrophenyl)-ethyl-ester[4]. Myeloperoxidase-based imaging is used for slow dividing myeloid cells[2], and fluorescence-based detection of cells labelled with fluorescent dyes. BLI for the detection of active MPO requires the injection of luminol i.p. the substrate of active MPO. For the detection of cells or vector system within certain cells, fluorescent dyes can be used. We have previously used an RFP containing vector in ovarian cancer cells that allowed us to detect a knockdown vector system *in vivo*[5].

PET imaging

[^{68}Ga]NODAGA-cyclo(Arg-Gly-Asp-D-Phe-Lys) is synthesized and labelled as previously described. This peptide is structurally similar to Cilengitide and has a comparable affinity to αv integrins as Cilengitide. 6 to 12 MBq/0.6 nmol/0.1 ml of [^{68}Ga]NODAGA-c(RGDfK) are injected via lateral tail vein and afterwards the mice are scanned for 30min using the small animal PET scanner microPET Focus 120 (Concorde Microsystems). PET images can be reconstructed with OSEM2D algorithm (2D Ordered Subset Expectation Maximization) provided by the scanner software. The resolution of the images ranges between 1.1 and 2.5mm depending on the distance from the center of the field of view. Image counts/pixel/second were calibrated to activity concentrations (Bq/mL) by measuring a ^{68}Ga cylindrical phantom filled with a known concentration of radioactivity.

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Microfluidics and Lab-on-a-Chip

Photograph: Leopoldina

HEAD Prof. Dr. Roland Zengerle

METHODS

We offer miniaturization, integration, parallelization, and automation of complex biochemical assays on low-cost polymer substrates by applying microfluidics and microengineering. Typical applications are point-of-care diagnostics, professional diagnostics, and industrial process control. We implement assays either on our validated lab-on-a-chip platforms LabDisk, and/or LabTube and provide the following specific support:

- Layout for microfluidic assay integration
- Simulation for straightforward optimization
- Short turnaround times for prototyping by micro-milling and micro-thermoforming
- Easy upscaling of production numbers on pilot line
- Versatile instruments for assay processing and readout

We provide all steps, from specifications, microfluidic design, simulation, prototyping of disposables including preloading of a multitude of reagents as well as functional testing.

Examples of integrated assays

- Nucleic acid-based tests (e.g. MRSA, Bacillus anthracis, various food pathogens)
- Immunoassays (e.g. IL8, estradiol)
- Enzymatic tests (e.g. cholesterol)
- Sample preparation (e.g. DNA extraction)
- Automation of laboratory tasks

Prototyping and Production of disposables

- Short turnaround times by precision micro-milled polymer masters and micro-thermoforming
- Replication in polymer foils or polymer substrates (diameters up to 130 mm)
- From small batch series of 10 pieces up to 200,000 pieces/year Reagent pre-loading

Reagent pre-loading

- Fully automated dispensing of up to 16 different reagents (5 µl to 500 µl)
- Air-drying of reagents for stabilization and long-time storage
- Automatic release of liquid buffers via stick-packs
- Contamination free handling of positive and negative controls

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- Detailed analysis of liquid behaviour, diffusion, or binding kinetics
- Design optimization of microfluidic structures

Instruments

For the operation of our LabDisk and LabTube assay implementations, we offer generic instruments (LabDisk player, centrifuges) which can be tuned to fit our customers' needs and cost requirements.

LabDisk for fully automated sample-to-answer point-of-care testing.
Photograph by: Bernd Müller Fotografie

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BIOSS members at the Retreat 2015 in Schluchsee in the Black Forest. Photograph by: Katrin Albaum/BIOSS

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