Developments in Advanced Microscopy and Spectroscopy Methods for Medicine

780. WE-Heraeus-Seminar

12 – 16 February 2023

at the Physikzentrum Bad Honnef, Germany

WILHELM UND ELSE
HERAEUS-STIFTUNG

Introduction

The Wilhelm und Else Heraeus-Stiftung is a private foundation that supports research and education in science with an emphasis on physics. It is recognized as Germany's most important private institution funding physics. Some of the activities of the foundation are carried out in close cooperation with the German Physical Society (Deutsche Physikalische Gesellschaft). For detailed information see https://www.we-heraeus-stiftung.de

Aims and scope of the 780. WE-Heraeus-Seminar:

Diagnostic techniques in medicine are dominated by analysis of body fluids, by tissue staining for optical microscopy, and by the range of omics approaches. However, these methods represent only a part of the plethora of biophysical methods on biological systems that are continuously being developed. Accordingly, more methods need to be translated into medical application for diagnosis considering the modern challenges of individualized medicine. Ideally, these approaches should be non-invasive, applicable to living systems and yield complementary information to existing standard procedures.

In this seminar, we bring together leading experts in modern, evolving fields such as super-resolution microscopy, near-field microscopy and in-cell spectroscopy with colleagues from medicine to discuss latest advances in biophysical techniques and their possible application to medical questions. Challenges remaining for translation into routine procedures will be discussed.

Scientific Organizers:

Prof. Dr. Tilman Kottke — Universität Bielefeld, Germany
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Introduction

Venue:

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Registration:

Elisabeth Nowotka (WE Heraeus Foundation)
at the Physikzentrum, reception office
Sunday (16:00 h – 21:00 h) and Monday morning

Program

Program

Sunday, 12 February 2023

16:00 – 20:00	Registration	
18:00	*BUFFET SUPPER and informal get-together*	
19:30	Scientific organizers	**Welcome words**
19:40	Hermann Einsele	**tba**

Monday, 13 February 2023

08:00	*BREAKFAST*	
08:55 – 09:10	Joachim Heberle	**Introduction**
09:10 – 10:00	Marloes Groot	**Translation of higher harmonic generation microscopy into the clinic for tumor tissue assessment**
10:00 – 10:50	Leif Schröder	**Hyperpolarized Xenon NMR for exploring molecular host cavities and advancing MR imaging**
10:50 – 11:20	*COFFEE BREAK*	
11:20 – 11:40	Jer-Shing Huang	**Chiral structured illumination microscopy for simultaneous imaging of chiral and achiral domains**
11:40 – 12:30	Katerina Kanevche	**Infrared nanoscopy and tomography of intracellular structures: glimpse inside cells with infrared light**
12:30	*LUNCH*	

Program

Monday, 13 February 2023

14:00 – 14:50	Jan Becker	**Unlabeled detection of biomolecules through mass photometry**
14:50 – 15:40	Werner Mäntele	**Novel infrared techniques for medical applications**
15:40 – 16:00	Karsten Niehaus	**Imaging small molecules in tissues and single cells by Mass Spectrometry Imaging (MSI)**
16:00 – 16:30	*COFFEE BREAK*	
16:30 – 18:30	**Poster session**	
18:30	*DINNER*	

Program

Tuesday, 14 February 2023

08:00	*BREAKFAST*	
08:55 – 09:10	Markus Sauer	**Introduction**
09:10 – 10:00	Gabriele Kaminski Schierle	**A small molecule drug inhibits Aβ1-42 aggregation and cellular thermogenesis.**
10:00 – 10:50	Mike Heilemann	**Exploring the inner workings of cells with super-resolution fluorescence microscopy**
10:50 – 11:00	**Conference Photo** (in the front of the lecture hall)	
11:00 – 11:20	*COFFEE BREAK*	
11:20 – 11:40	Christina Verbruggen	**dSTORM imaging of chimeric antigen receptors on the T cell membrane**
11:40 – 12:30	Nils Brose	**Dynamic regulation of presynaptic function and plasticity in health and disease**
12:30	*LUNCH*	

Program

Tuesday, 14 February 2023

14:00 – 14:50	Jennifer Lippincott-Schwartz	**Looking under the hood of cells: from single molecule dynamics to whole cell organelle reconstructions**
14:50 – 15:40	Gerhard Schütz	**Following T cell antigen recognition molecule by molecule**
15:40 – 16:00	Gerti Beliu	**Bioorthogonal click chemistry enables site-specific fluorescence labeling for quantitative super-resolution imaging**
16:00 – 16:30	*COFFEE BREAK*	
16:30 – 17:20	Silvio Rizzoli	**Expansion microscopy at one nanometer resolution**
17:20 – 17:40	Christian Franke	**Unraveling the nanoscale Fate of mRNA Vaccines by multi-colour dSTORM**
17:40 – 18:00	Stefan Jorda	**About the Wilhelm and Else Heraeus-Foundation**
18:30	*HERAEUS DINNER* *(social event with cold & warm buffet with complimentary drinks)*	

Program

Wednesday, 15 February 2023

08:00	*BREAKFAST*	
08:55 – 09:10	Tilman Kottke	**Introduction**
09:10 – 10:00	Olav Schiemann	**Pulsed dipolar EPR spectroscopy: Following conformational changes of biomacromolecules with time and in cells**
10:00 – 10:50	Philipp Selenko	**Looking at proteins in live cells with atomic resolution: From science fiction to science reality**
10:50 – 11:20	*COFFEE BREAK*	
11:20 – 11:40	Lukas Gött-Zink	**In-cell infrared difference spectroscopy on blue light receptors in living cells**
11:40 – 12:30	Ute Neugebauer	**Raman spectroscopy as emerging method in clinical spectroscopic diagnostic of infections**
12:30	*LUNCH*	

Program

Wednesday, 15 February 2023

14:00 – 14:50	Marc Baldus	**Probing the conformational landscape of biomolecules in situ using NMR spectroscopy**
14:50 – 15:40	Martina Meinke	**Application of electron paramagnetic resonance (EPR) spectroscopy in dermatology**
15:40 – 16:00	Ulrike Alexiev	**Advanced biophysical visualization methods for nanomedicine and theranostics**
16:00 – 16:30	*COFFEE BREAK*	
16:30 – 17:20	Thomas Huser	**Multiscale fluorescence imaging of the liver - from the mesoscale to the nanoscale**
17.20 – 17.40	Sven Thoms	**High-end microscopy of peroxisomes**
17:40 – 18:30	**Discussion and outlook**	
18:30	*DINNER*	

Thursday, 16 February 2023

08:00	*BREAKFAST*

End of the seminar and departure

Posters

Posters

Jan Vincent Arafiles	**Next generation cell-penetrating peptide additives**
Federico Baserga	**In Situ ATR FTIR spectroscopy as a benchmark for the catalytic reactions of complex IV**
Julian Düwel	**Imaging the uptake and effects of microplastics on organisms by coherent raman scattering microscopy**
Jacobo Gómez-González	**Improving cell specificity of cell-penetrating peptide additives**
Paul Greife	**Tracking water oxidation through time-resolved FTIR spectroscopy**
Caspar Anton Heubach	**The dHis-Cu^{2+}(NTA) motif: Site specificity of Cu^{2+}(NTA)-binding and evaluation of pulsed dipolar EPR data involving this motif**
Jer-Shing Huang	**Chiral structured illumination microscopy for simultaneous imaging of chiral and achiral domains**
Andrey Iljin	**Non-uniform holographic grating as a virtual noninvasive probe**
Pit Langner	**Probing protein dynamics using quantum cascade laser based surface enhanced infrared absorption spectroscopy**
Silke Lohan	**EPR spectroscopy as read-out system of redox homeostasis in blood plasma with UVA irradiation as a stressor**
Sarah Mäusle	**Advancements in Time-Resolved Single-Frequency Infrared Spectroscopy on Photosystem I and II**
Henning Ortkraß	**High-speed TIRF and 3D super-resolution structured illumination microscope with large field of view based on fiber optic components**

Posters

Matthias Plessner	**Actin' in cell division**
Mattia Saita	**Non-invasive blood glucose measuring device: From lab to bed side**
Luiz Schubert	**Protein conformational and protonation dynamics monitored by single-shot IR spectroscopy**
Jasmin Celine Schürstedt	**Three-dimensional optical projection tomography of biomedical tissues**
Sylvia Marie Steinecker	**3D short-wave infrared (SWIR) imaging – developing an optical projection tomography (OPT) setup**
Linda Stelz	**Intracellular visualization of SARS-CoV-2 RNA by super-resolution microscopy**
Corinna Strothenke	**Perfusion-induced FTIR difference spectroscopy on human ABC transporters**
Maria Francesca Vicino	**Following conformational changes in *Lbu*Cas13a from apo to ternary cr- and target RNA bound state with EPR spectroscopic distance measurements**
Daniela Zamudio Díaz	**Skin safety of 233 nm Far-UVC irradiation in ex vivo human skin and healthy humans**

Abstracts of Talks

(in chronological order)

Translation of higher harmonic generation microscopy into the clinic for tumor tissue assessment

Marie Louise Groot

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Surgeons ultimately rely on tactile information or visual inspection when they need to decide which tissue to remove. Depending on tumor and type of surgery, tumor positive margins occur in 10—35 % of surgeries. In addition, surgeries may be hampered or lead to loss of function because nerve tissue or other functionally important parts are close to the tumor location and cannot be well recognized. Similarly, when taking biopsies for diagnosis, multiple biopsies are taken to increase the chance for representative tissue, resulting in prolonged procedures, patient discomfort, and increased risk of complications. Clearly, there is a pressing need for a technology that can aid in quick assessment and diagnosis of excised tissue.

Higher harmonic generation (HHG) microscopy is a novel promising imaging technique that meets these requirements. This technique is non-invasive, label-free, and provides 3D images with a high, sub-cellular resolution, within seconds. Before, we demonstrated that HHG microscopy can generate high quality images of freshly excised unprocessed lung and brain tissue, in less than a minute with information content comparable to that of the gold standard, histopathology [1,2].
In our most recent study we brought mobile HHG microscopes into two hospitals to image freshly excised biopsies. The results so far show that HHG microscopy enables real-time 3D imaging of the biopsies and reveals pathological hallmarks which enable making a quick preliminary diagnosis. Here, I will present our latest results on lung, thyroid, pancreas and pediatric tumor tissues.

Finally, automatic image analysis would eliminate the need for a pathologist to be present in the endoscopy suite or operation theatre. I will discuss our progress towards using convoluted neural networks in the assessment of brain and lung tissue.

1. van Huizen, L. M. G., Radonic, T., van Mourik, F., Seinstra, D., Dickhoff, C., Daniels, J. M. A., Bahce, I., Annema, J. T., and Groot, M. L. (2020) Compact portable multiphoton microscopy reveals histopathological hallmarks of unprocessed lung tumor tissue in real time, *Translational Biophotonics*, e202000009.
2. Zhang, Z. Q., de Munck, J. C., Verburg, N., Rozemuller, A. J., Vreuls, W., Cakmak, P., van Huizen, L. M. G., Idema, S., Aronica, E., Hamer, P. C. D., Wesseling, P., and Groot, M. L. (2019) Quantitative Third Harmonic Generation Microscopy for Assessment of Glioma in Human Brain Tissue, *Advanced Science 6*

Hyperpolarized Xenon NMR for Exploring Molecular Host Cavities and Advancing MR Imaging

L. Schröder[1]

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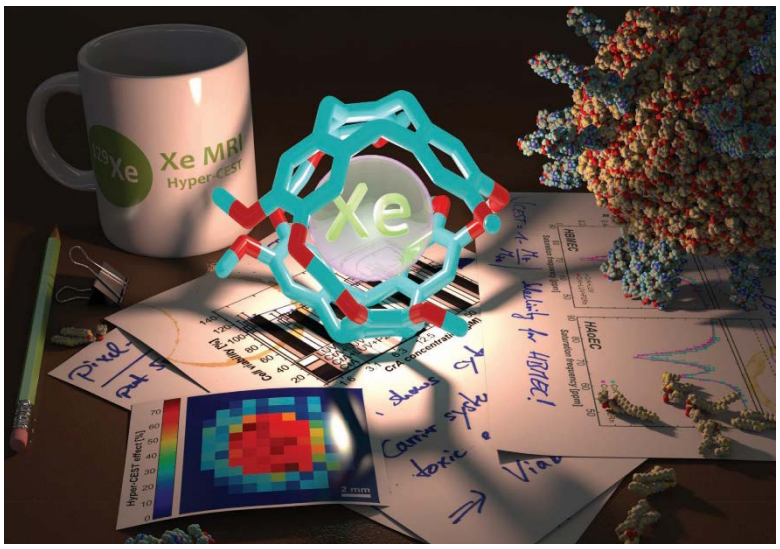
Nuclear Magnetic Resonance (NMR) suffers from low sensitivity for inductive detection of thermally polarized spin ensembles. On the other hand, the high molecular specificity of NMR makes the method an important tool in biophysics, chemistry, and biomedical imaging. Hyperpolarized spin systems can be achieved with dissolved noble gases like ^{129}Xe (spin ½) to provide 10'000-fold improved sensitivity over extended periods of time.[1] In combination with saturation transfer techniques, reversibly bound hyperpolarized xenon gives insights into exchange kinetics of various host-guest complexes. This talk will give an overview of studies investigating the affinity of dissolved Xe for various hosts like biogenic hollow protein structures[2] with attoliter volumes or synthetic hosts for trapping individual Xe atoms.[3,4] Both strategies find applications in the spectroscopic investigation of exchange kinetics of host-guest systems and in the design of ultra-sensitive magnetic resonance imaging agents.



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[1] D. A. Barskiy, et. al. Chem. – Eur. J. **23**, 725–751 (2017)

[2] M. Kunth, G. J. Lu, C. Witte, M. G. Shapiro, L. Schröder, ACS Nano, **12**, 10939–10948 (2018)

[3] J. Jayapaul et. al. Nat. Commun. **11** (2022).

[4] J. O. Jost, L. Schröder, NMR Biomed., e4714 (2022).

Chiral structured illumination microscopy for simultaneous imaging of chiral and achiral domains

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The ability to fast image chiral domains at sub-diffraction limited resolution is valuable for understanding the spatial distribution of chiral species and may find applications in drug design, material sciences, and biomedical imaging. Previously, we proposed a new chiral domain imaging modality, named "Chiral Structured-illumination Microscopy (Chiral SIM)". This method is based on optical chirality engineering, fluorescence-detected circular dichroism, and structured illumination microscopy. In chiral SIM, the optical chirality of the illumination is structured and the circular dichroism-dependent fluorescence from chromophores near molecular chiral domains is detected. With image reconstruction, the spatial distribution of chiral domains with sub-diffraction limited resolution can be obtained [1]. Here, we show that chiral SIM can be easily extended to "Double SIM", which enables simultaneous imaging of achiral and chiral domains at a sub-wavelength resolution [2]. In double SIM, the illumination field is spatially structured both in the intensity and optical chirality so that moiré effects can be concurrently generated on the achiral and chiral fluorescent domains of a sample. This allows for down-modulating the high spatial frequency of both domains at the same time and thus provides subwavelength details after image reconstruction. We introduce the working principle and theoretically demonstrate the feasibility of these methods.

References

[1] S.-Y. Huang, J. Zhang, C. Karras, R. Förster, R. Heintzmann, J.-S. Huang, ACS Photonics, Journal **8**, 130-134 (2020)

[2] J. Zhang, S.-Y. Huang, A. K. Singh, and J.-S. Huang, Optics Letters **46**, 4546-4549 (2021)

Infrared nanoscopy and tomography of intracellular structures: glimpse inside cells with infrared light

Katerina Kanevche[1], David J. Burr[2], Dennis J. Nürnberg[3], Pascal K. Hass[4], Andreas Elsaesser[2], Joachim Heberle[1]

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Abstract

Infrared (IR) microscopy and spectroscopy, while being sensitive to the samples' chemical composition, suffer from poor lateral resolution due to diffraction. Therefore, imaging of the cellular inner structure in the IR range of the electromagnetic spectrum proves to be challenging. An approach to overcome this limitation is by using scattering-type near-field optical microscopy (sSNOM) and nano-Fourier transform infrared (nanoFTIR) spectroscopy, achieved by combining the high spatial resolution of atomic force microscopy (AFM) and the chemical sensitivity of IR absorption. We were able to resolve the subcellular structure of *C. reinhardtii* and assign the IR absorption of various organelles to molecular vibrations with spatial resolution of 20 nm [1]. For instance, this allowed to resolve the microtubular structure of the algal flagellum. The necessity and power of chemical imaging was demonstrated by scanning the nuclear area, where several nuclear bodies were distinguished in the sSNOM images while remaining hidden in the AFM topography. Finally, a stack of sSNOM images, obtained by sequential scanning of serial sections, was used to reconstruct a three-dimensional image. Thus, we demonstrate that sSNOM tomography allows visualizing three-dimensional intracellular structures at nanometer resolution where the contrast originates from molecular vibrations of chemical bonds.

1. Kanevche, K., et al., *Infrared nanoscopy and tomography of intracellular structures.* Commun Biol, 2021. **4**(1): p. 1341.

Unlabeled detection of biomolecules through mass photometry

Jan Becker[1,2] and Philipp Kukura[1,2]

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[2] Physical and Theoretical Chemistry Laboratory, University of Oxford, UK

Light scattering-based microscopy has made significant progress over the past decade, reaching the single molecule level both for resonant and non-resonant detection. We have been approaching the challenge of ultrasensitive detection through mass photometry (MP) [1]. MP enables not only imaging of single biomolecules in solution without labels, it does so with sufficient accuracy and precision to measure the molecular mass of individual species resulting in high levels of resolution. I will introduce MP in the context of interference techniques more broadly, and explain the key technological advances enabling the current levels of detection sensitivity and measurement precision [2]. I will employ Fourier optics to model light scattering from nanoscopic objects (including shape) and image formation by interference with background light. Coupling this model with atomic force microscopy of glass substrates commonly used in mass photometry [3] results in a theoretical description in close agreement with experiment in terms of both imaging background and commonly encountered signal magnitudes for polypeptides with appropriate mass scaling. Taken together, these results establish mass photometry as an extremely powerful, solution-based, label-free, yet single molecule method to quantify and thereby study biomolecular structure and interactions [4].

References

[1] G. Young, N. Hundt, D. Cole, A. Fineberg, J. Andrecka, A. Tyler, A. Olerinyova et al., Science 2018, **360**, 423 – 427
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[4] N. Hundt, D. Cole, M.F. Hantke, J.J. Miller, W.B. Struwe, and P. Kukura, Science advances 2022. **8**(35)

Novel Infrared Techniques for Medical Applications

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Mid-IR spectroscopy has proven to be a suitable tool for the *ex vivo* analysis of body fluids such as blood, plasma, interstitial fluid, urine or dialysis fluid [1-3]. With the advent of the quantum cascade laser (QCL), powerful narrow-band single wavelength IR emitters, multi-wavelength emitter arrays, or, with an external cavity (EC), widely tunable EC-QCLs are available. In the pulsed mode, QCL are an ideal IR light source for photometric measurement of IR radiation absorbed in skin or in tissues in combination with photoacoustic or photothermal detection. The most attractive application is the non-invasive measurement of glucose in the interstitial fluid of skin for the (self)monitoring of blood glucose for diabetes patients.

We present here our developments in QCL applications for the measurement of skin parameters and body fluids *in vitro* and *in vivo*. A photothermal detection method has been developed that combines an Internal Reflection Element (IRE) with PhotoThermal Detection (IRE-PTD) [4]. The latter allows the detection of IR absorbance using visible light and appears to be ideal for the analysis of human skin, in particular with a depth profiling on the basis of different modulation frequencies.

Based on an earlier laboratory setup, we have developed and CE certified a shoe-box sized multi-user device (“D-Base”) that contains all optical and electronic components and that is controlled by a tablet PC. This device has been clinically validated in a test with healthy and diabetic volunteers; its accuracy is close to the standard invasive pricking method with tests strips or to the minimally invasive continuously measuring glucometers (CGM) [5].

Based on the IR technology in D-Base, a miniaturized personal glucometer (“D-Pocket”), about the size of a smartphone, is currently developed by DiaMonTech. It uses a multi-wavlength QCL array instead of a tunable EC QCL combined with photothermal detection. We expect D-Pocket to be complete by Q2 2023 and, after CE certification and clinical validation, ready for the market.

References

[1] Hoşafçı et al. (2007), Analytical and Bioanalytical Chemistry 387:1815–1822
[2] Roth et al. (2012), Analytical and Bioanalytical Chemistry 403(2):391-399
[3] Pleitez et al. (2012), Spectrochimica Acta Part A 85:61– 65
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Imaging small molecules in tissues and single cells by Mass Spectrometry Imaging (MSI)

Hanna Bednarz[1], Judith Neumann[2], Maria Sievers[2], Christian Pilger[3], Henning Hachmeister[3], Thomas Huser[3], Karsten Niehaus[2]

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Light- and electron microscopy allows to analyze the structure of single cells, tissues, and organs. DNA, proteins, and saccharides can be localized by different approaches. Nevertheless, techniques to localize small molecules are missing. Knowing the physiology of a microbe, plant, animal, or human being is essential to understand its development, environmental adaptation, and probably the transition from healthy to diseased. Mass Spectrometry Imaging (MSI) adds a new dimension to the understanding of physiological processes by localizing the metabolites and xenobiotics to organs, tissues, and even single cells. MALDI-MSI offers the advantage of high spatial resolution (up to 5 µm) with high speed (20 pixel / sec.) in scanning the sample. This is essential if it comes to applications in digital pathology where light microscopy and MSI is combined. Novel approaches aim to use multimodal imaging to improve the scientific significance of MSI data. Examples for biomarker discovery in tumor research and vascular diseases will be given.
Thin sectioning and classical histochemistry of human artery resulted in the identification of arteriosclerotic alterations by classical light microscopy. Incorporation of lipids was analysed by CARS-microscopy. MALDI-TOF-MSI of the human arteriosclerotic coronary artery resulted in the identification of 160 mass channels that arose from the analysed tissue. Calcification-specific biomarkers could be specified by their localization.
Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor. To determine the metabolic alterations between tumor and peritumoral tissue in human GBMs, mass spec- trometry imaging (MSI) was performed on thin sections from 25 resected tumors. Increased levels of purine and pyrimidine metabolism compounds in GBM areas indicate the high energy demand. In accordance with these results, enhanced abundances of lactate and glutamine were detected. Moreover, decreased abundance of N-acetylaspartate, a marker for neuronal health, was measured in tumor areas. Obtained metabolic information could potentially support and personalize therapeutic approaches, hence emphasizing the suitability of MSI for GBM research.
The last will demonstrate how MALDI-MSI, in combination with fluorescence microscopy, can be used to analyze the behavior of cell populations as the basis of single cell metabolomics.

References:

(1) Bednarz & Niehaus in MALDI Mass Spectrometry Imaging; RSC 2021
(2) Kampa et al., Neuropathology. 2020 Dec;40(6):546-558
(3) Neumann et al., J Cancer Res Clin Oncol. 2022 Feb;148(2)

A small molecule drug inhibits Aβ1-42 aggregation and cellular thermogenesis.

Chyi Wei Chung[1], Suil Collins[1], David Spring[1], Florian Hollfelder[1], Clemens F Kaminski[1], and Gabriele S. Kaminski Schierle[1]

[1]University of Cambridge, Cambridge, UK

The aggregation of amyloid β (1-42), Aβ42, is a hallmark of Alzheimer's disease and inhibiting this process is thus a promising strategy for therapeutic intervention. To date, however, no efficacious inhibitors have been identified, despite the efforts of more than 200 major drug development campaigns reported so far. Progress in the field is severely hampered by limitations in current compound screening technologies and *in vivo* validation assays.

In this talk I will report on a novel screening platform enabled by an aggregation sensor developed in our group that makes use of Fluorescence lifetime imaging, FLIM, as a powerful readout of aggregation state. Our assay combines optical with microfluidic technologies and circumvents issues that plague conventional assays, such as lack of reproducibility, high cost and artefactual read-outs. I will show that lifetime sensing can be used also to dynamically monitor peptide aggregation in cells and in a live *C. elegans* disease model. The system accelerates hit-to-lead strategies, lowering attrition rates and expediting *in vivo* screening. I demonstrate the power of the system with a pilot screen of 445 compounds, producing a new inhibitor that was found to prevent Aβ self-assembly *in vitro* as well as in cellular and whole organism models of disease.

In parallel, we conduct molecular level research on the aggregation dynamics of amyloidogenic proteins. Although Aβ42 elongation is an exothermic process, nothing was known on how this affects cells in which amyloidogenesis takes place. We were able to show that Aβ42 aggregation leads to thermogenesis, a rise of intracellular temperature. This may cause cellular stress which itself may be a promoter of aggregation. I will report on a on intracellular thermometry measurements using fluorescent polymeric thermometers (FPTs) to show that Aβ42 aggregation in live cells leads to an increase in cell-averaged temperatures. This rise in temperature is mitigated upon treatment with the small molecule inhibitor described above.

Exploring the inner workings of cells with super-resolution fluorescence microscopy

M. Heilemann[1]

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Super-resolution fluorescence microscopy has evolved into a powerful tool for optical structural cell biology. Providing a spatial resolution at the protein size scale, it is beginning to revolutionize our understanding of cell biology.
Our work focuses on two main challenges: the extraction of quantitative information from imaging data, with the purpose of characterizing the number of molecular constituents in densely-packed protein assemblies that are so-far inaccessible to direct visualization; the contextualization of biomolecular structures in a cell through multi-target imaging. For the first part, we developed kinetics-assisted quantitative single-molecule localization microscopy (qSMLM), which retrieves molecule numbers from fluorescence emission signatures [1]. We validate this approach for various fluorophore labels and flavors of SMLM, and apply it to reveal the function-dependent oligomerization of membrane receptors TNFR1 [2], TLR4 and MET. For the second part, we explore the use of non-covalent and weak-affinity protein labels for many-target fluorescence imaging [3, 4]. This dynamic labeling approach minimizes photobleaching, which minimizes information loss in 3D imaging, and enables long-time live cell microscopy and imaging of large fields of view. It further bypasses the 'spectral barrier' of fluorescence microscopy and facilitates targeting many molecules through repetitive washing steps. This concept is generalizable for various (super-resolution) fluorescence microscopy methods [5, 6].

References

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[5] Glogger M et al., Angew Chem **60**, 6310-6313 (2020).
[6] Glogger M et al., ACS Nano **16**, 17991-17997 (2022).

*d*STORM imaging of Chimeric Antigen Receptors on the T cell membrane

C.M. Verbruggen[1], L. Gehrke[2], A. Ghosh[1], T. Nerreter[2], M. Hudecek[2], H. Einsele[2], M. Sauer[1]

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Chimeric Antigen Receptor (CAR)-T cell therapy is a promising antitumor therapy, which can be used for the treatment of a large variety of cancers. Despite countless developments in the CAR-T cell field, the dynamics of CAR expression and distribution on the surface of engineered T cells remains elusive. This is largely due to the fact that a reliable way to detect CARs with single-molecule precision is currently not available.

We present a way of direct CAR detection in a receptor-intrinsic manner without inclusion of additional tags. This allowed us to visualize the CAR on the T cell surface at single molecule resolution using dSTORM, enabling a precise quantification of CAR expression. We found that our staining method is reliable on Jurkat cells as well as primary CD4+ and CD8+ T cells and tested it for several different CAR constructs. We discovered that CAR expression is highly variable on a cell-to-cell basis and that a certain percentage of CARs are clustered, independent of CAR construct, T cell type, donor, transgene delivery method and expression level. Confocal and live-cell lattice light-sheet fluorescence imaging of CAR-T cells interacting with tumor cells revealed that CARs accumulate in clusters at contact areas and form multifocal immunological synapses.

Furthermore, we correlated surface expression with in vitro assays for different transgene-delivery methods and found differences in CAR expression, cytolytic activity and activation-induced cell death between knock-in generated and lentivirally transduced CAR-T cells with varying target antigen expression on tumor cells. Hence, our data suggest that not only target antigen density and tumor entity but also CAR surface expression and transgene delivery method should be considered for an optimal treatment outcome.

Dynamic regulation of presynaptic function and plasticity in health and disease

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The process of synaptic vesicle priming is an essential key determinant of synapse function, strength, and plasticity because it maintains a pool of readily releasable vesicles at any given time and determines the time course of synaptic fatigue and recovery, e.g. upon exhaustion of readily releasable vesicles during and after phases of high synaptic activity. The corresponding forms of synaptic short-term plasticity determine multiple complex brain functions, from sensory adaptation to working memory. Munc13s execute synaptic vesicle priming by regulating the assembly of fusogenic SNARE complexes. They are regulated by three major pathways, involving (i) calcium-calmodulin signaling via dedicated amphipathic calcium-calmodulin binding sites, (ii) diacylglycerol signaling via C1 domains, and (iii) calcium-phospholipid signaling via C2 domains. We studied the functional relevance of these regulatory pathways in various synapse types and found that calcium-dependent Munc13-regulating pathways are major determinants of synaptic short-term plasticity, synapse endurance, and synaptic fidelity. I will discuss these data in the context (i) of the combined role of signaling pathways that target presynaptic function, (ii) of the role of Munc13 priming proteins in determining the unique features of regulated exocytosis at nerve cells synapses, and (iii) of the role of Munc13 mutations in neuropsychiatric disorders.

Looking under the hood of cells: from single molecule dynamics to whole cell organelle reconstructions

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Powerful new ways to image the internal structures and complex dynamics of cells are revolutionizing cell biology and bio-medical research. In this talk, I will focus on how emerging fluorescent technologies are increasing spatio-temporal resolution dramatically, permitting simultaneous multispectral imaging of multiple cellular components. In addition, results will be discussed from whole cell milling using Focused Ion Beam Electron Microscopy (FIB-SEM), which reconstructs the entire cell volume at 4 voxel resolution. Using these tools, it is now possible to begin constructing an “organelle interactome”, describing the interrelationships of different cellular organelles as they carry out critical functions. I will also present an additional tool, single particle tracking, describing how it can be used to characterize the dynamics of organelle contact sites and the behavior of tethering proteins. Together, the new tools are revealing new properties of organelles and their trafficking pathways, and how disruptions of their normal functions due to genetic mutations may contribute to important diseases.

Following T cell antigen recognition molecule by molecule

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T-cells readily detect the presence of even a single antigenic peptide/MHC complex (pMHC) among thousands of endogenous pMHCs via T-cell receptors (TCRs) on the surface of antigen-presenting cells. The mechanisms underlying this phenomenal sensitivity have remained elusive. Recent studies suggest that the topography of the immunological synapse formed between the T cell and the antigen-presenting cell is of pivotal importance for these processes. We hence were first interested, how the TCR is distributed within the immunological synapse. For this, we used single molecule localization microscopy in combination with supercritical angle detection to localize single TCR molecules at an isotropic precision below 15nm [1, 2]. Second, researchers speculated that mechanical forces could be instrumental for the high specificity and sensitivity of the T cell response. Force magnitude, spread, and temporal behavior, however, are still poorly defined. We developed a calibrated FRET-based sensor equipped either with a TCR-reactive single chain antibody fragment or peptide-loaded MHC. The sensor was tethered to planar glass-supported lipid bilayers and informed most directly on the magnitude and kinetics of TCR-imposed forces at the single molecule level. From the single molecule FRET signals we quantified the magnitude of tensile forces exerted by T cells via single TCR molecules. In addition, the data allowed us to draw conclusions on the directionality of the observed forces, as well as on the pulling speed.

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Abstract

Abstract (780. WE-Heraeus-Seminar)

"Bioorthogonal Click Chemistry Enables Site-specific Fluorescence Labeling for Quantitative Super-Resolution Imaging"

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Optical nanoscopy has contributed significantly to our understanding of biological structures. Although steadily emerging fluorescence techniques are constantly improving spatio-temporal resolution and thus enable visualization of biological processes in real time, there are still a number of challenges to overcome [1].
Since the density of fluorophores controls the achievable structural resolution, efficient and specific labeling with fluorescent probes is a decisive factor in super-resolution microscopy techniques. Despite recent progress in the development of new fluorophores with superior photophysical properties, specific and efficient labeling of the molecule of interest with minimal linkage error remains a significant challenge. As the field moves towards higher spatial resolution, the effective size of the label will be the main limiting factor of super-resolution microscopy, demanding the development of efficient labeling methods with small dyes, which can be site-specifically and quantitatively attached to a protein of interest with low linkage error [2-3].
Site-specific introduction of unnatural amino acids (uAA) into proteins of interest followed by bioorthogonal click chemistry with tetrazine-dyes represents a broadly useful possibility to overcome current limitations and enable high-end fluorescence imaging with organic dyes. A particularly promising type of uAA include strained alkenes, such as trans-cyclooct-2-ene (TCO), that can react with a 1,2,4,5-tetrazine in an ultrafast, specific and bioorthogonal click reaction.
Additionally, the tetrazine moiety can elicit substantial quenching of many fluorescent dyes (e.g. red-absorbing oxazines and rhodamine derivatives) and thus be utilized for improved live-cell labeling and quantitative super-resolution imaging experiments under physiological and wash-free conditions. Furthermore, this powerful tool can be applied to improve protein-protein interaction (PPI) assays [4-5], making them faster, more accurate and easier to implement.

In conclusion, new labeling techniques will enable previously impossible experiments with unseen reactivity and specificity while preserving biological function. Advances of optical microscopy in biological science will depend crucially on advances in sample preparation and labeling.

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Expansion microscopy at one nanometer resolution

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Fluorescence imaging is one of the most versatile and widely-used tools in biology. Although techniques to overcome the diffraction barrier were introduced more than two decades ago, and the nominal attainable resolution kept improving to reach single-digit nm, fluorescence microscopy still fails to image the morphology of single proteins or small molecular complexes, either purified or in a cellular context. Here we report a solution to this problem, in the form of one-nanometer expansion (ONE) microscopy [1]. We combined the 10-fold axial expansion of the specimen (1000-fold by volume) with a fluorescence fluctuation analysis to achieve resolutions down to 1 nm or better. We have successfully applied ONE microscopy to image cultured cells, tissues, viral particles, molecular complexes and single proteins. At the cellular level, using immunostaining, our technology revealed detailed nanoscale arrangements of synaptic proteins, including a quasi-regular organisation of PSD95 clusters. At the single molecule level, upon main chain fluorescent labelling, we could visualise the shape of individual membrane and soluble proteins. Moreover, conformational changes undergone by the ~17 kDa protein calmodulin upon Ca2+ binding were readily observable. We could also image and classify molecular aggregates in cerebrospinal fluid samples from Parkinson's Disease (PD) patients, which represents a promising new development towards an improved PD diagnosis. ONE microscopy is compatible with conventional microscopes and can be performed with the software we provide here as a free, open-source package. This technology bridges the gap between high-resolution structural biology techniques and light microscopy, and provides a new avenue for discoveries in biology and medicine.

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Unraveling the nanoscale Fate of mRNA Vaccines by multi-colour dSTORM

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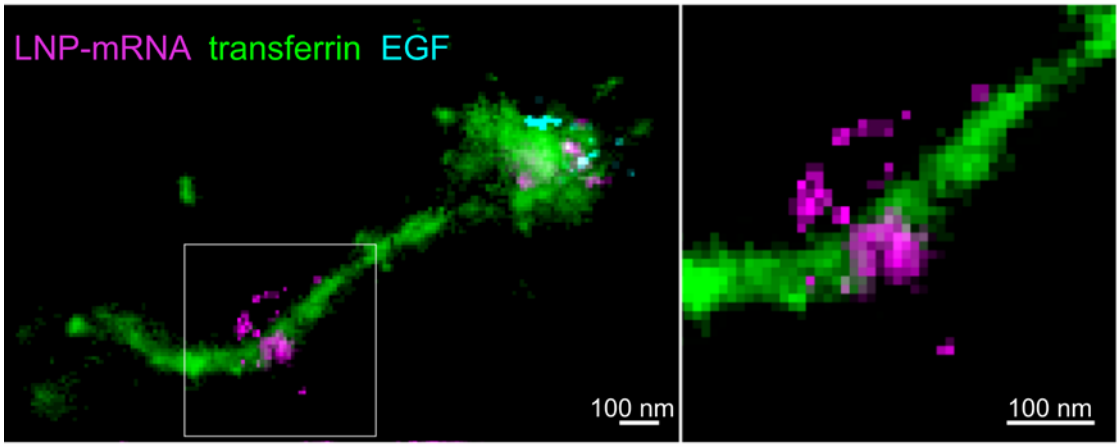
Delivery of exogenous mRNA using (lipid) nanoparticles (LNPs) is a promising strategy for therapeutics. However, a bottleneck remains in the poor understanding of the parameters that correlate with endosomal escape versus cytotoxicity. To address this problem, we compared the (sub-) endosomal distribution of six LNP-mRNA formulations of diverse chemical composition and efficacy, similar to those used in mRNA-based vaccines, in primary human adipocytes, fibroblasts, and HeLa cells. Surprisingly, we found that total uptake is not a sufficient predictor of delivery, and different LNPs vary considerably in endosomal distributions. Prolonged uptake impaired endosomal acidification, a sign of cytotoxicity, and caused mRNA to accumulate in compartments defective in cargo transport and unproductive for delivery. In contrast, early endocytic/recycling compartments have the highest probability for mRNA escape. The nanoscale complexity of the endosomal network cannot be underestimated and calls for a thorough quantitative and super-resolution analysis to interpret their precise identities. By using triple-colour *d*STORM, we could resolve single LNP-mRNA within subendosomal compartments and capture events of mRNA escape from endosomal recycling tubules (see Figure) [1]. Escape efficiency arguably depends on the (targeted) distribution of NPs in various subcellular compartments. Our results change the view of the mechanisms of endosomal escape and define quantitative parameters to guide the development of mRNA formulations toward higher efficacy and lower cytotoxicity [2].



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Pulsed Dipolar EPR Spectroscopy: Following Conformational Changes of Biomacromolecules with Time and In Cells

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The function of biomacromolecules is linked to their structure and dynamics. Pulsed Dipolar Electron Paramagnetic Resonance Spectroscopy (PDS) in combination with site directed spin labeling is a versatile tool to resolve the involved conformational changes in proteins, nucleic acids and their complexes. In the talk, this will be demonstrated on example of CRISPR/Cas 13a. However, the PDS measurements are usually done in the frozen state, meaning that the time scale of the conformational change is lost. We therefore combined PDS with a Microsecond freeze HyperQuench (MHQ) setup by which we were able to resolve the movement of an α-helix with microsecond time and Angstrom length resolution. Finally, it is highly desirable to follow the conformational change under as natural conditions as possible, meaning within cells and at natural concentrations. We address this challenge by using a new type of label, i.e., trityl radicals, which are stable within cells and enable recording PDS data of biomacromolecule within cells down to nanomolar concentrations.

Looking at proteins in live cells with atomic resolution: From Science Fiction to Science Reality

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The past decade has seen tremendous advancements in multiple areas of *in situ* Structural Biology. That is, in methods and technologies that enable direct structural investigations of biological macromolecules in their native cellular settings. In these applications, spectroscopic techniques such as nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) methods, together with Förster resonance energy transfer (FRET) spectroscopy play fundamental roles in providing unique and complementary insights. In combination with cellular cryo-electron tomography (cryoET), in-cell cross-linking mass spectrometry (XL-MS), computational methods such as AlphaFold and high-resolution optical imaging, they define the toolkit that will shape the face of Structural Biology in the years to come.

Here, I present my vision for the Structural Biology Department of the future. I outline how these techniques and technologies may be best integrated to arrive at a comprehensive understanding of biological processes in their native cellular settings. Because the future of Structural Biology … is in the cell.

In-cell Infrared Difference Spectroscopy on Blue-light Receptors in Living Cells

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FT-IR difference spectroscopy is widely used for studying structural changes of proteins *in vitro* such as enzymes or receptors. However, conditions *in vitro* differ dramatically to those in cells and proteins can be affected by intracellular metabolites. Recently, we established an approach of in-cell infrared difference spectroscopy (ICIRD) to study the structural response of soluble photoreceptors in living bacterial cells with a protein copy number of ~300,000 in the transmission and attenuated total reflection (ATR) configuration. ICIRD on a light, oxygen, voltage (LOV) photoreceptor revealed remarkable differences in the activation mechanism between *in vitro* and in cells. These deviations were rationalized by emulating the intracellular environment *in vitro* [1]. Further, we extended ICIRD to a time-resolved method by applying the rapid-scan technique. The setup was constructed using the rapid-scan technique, a pulsed laser synchronized to the interferometer, and a home-build automatic sample changer yielding a time resolution of 7.6 ms on a LOV photoreceptor in *E. coli* cells.

In addition, ICIRD allows for the investigation of proteins that cannot be isolated. The plant cryptochrome pCRY from Chlamydomonas reinhardtii is a blue-light receptor containing a photolyase homology region (PHR) and an C-terminal extension (CCT). However, isolation of full-length pCRY fails due to degradation. With ICIRD we were able to study full-length pCRY in bacterial cells. Comparison of PHR and pCRY in cells revealed a shift of a beta-sheet signal in the presence of CCT, indicating an association of the CCT to the only beta-sheet in the PHR.

As a next step we switched the host system from bacterial cells to human cell lines with medical relevance. Human cells were cultivated on an ATR crystal inside an FTIR spectrometer using a home-built cell cultivation chamber. Adherent cell growth on the ATR crystal can be monitored in situ by signals of the cell components. Preliminary studies on PHR in human cells by ICIRD allowed us to resolve the photoreaction of the chromophore in PHR. Future plans include investigation of the structural response of receptors in living human cells.

ICIRD expands the range of in-cell methods by a non-invasive and label-free technique, which does not require any purification step and has a limitation in number of copies but no intrinsic limitation in protein size. The application of ICIRD on human cell lines might contribute to medical research.

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Raman spectroscopy as emerging method in clinical spectroscopic diagnostic of infections

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In Raman spectroscopy, the biological sample is illuminated with monochromatic laser light and the inelastically scattered light is analyzed in order to extract fingerprint-like information about the molecular composition of the sample. As this process is non-destructive and uses intrinsic chemical information of the sample without the need of any label, Raman spectroscopy offers high potential for application in medicine. Several applications have been reported and range from pathogen characterization (virus, bacteria, fungi), cell differentiation and tissue classification.
This contribution will focus on the application Raman spectroscopy for improved diagnosis of infections. This will involve spectroscopic characterization of immune cells to unravel the host response to infection [1-4], as well as the detection and characterization of the bacterial pathogen during intracellular infections [5].

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Acknowledgment
Financial support by the BMBF (CSCC, FKZ 01EO1502, Photonics Research Germany, LPI-BT4 13N15708), the EU (Grant No. 861122: “ImageIN” and CN611682 “HemoSpec”), Leibniz Society (InfectoOptics) and DFG (JBIL) are highly acknowledged.

Probing the conformational landscape of biomolecules in situ using NMR spectroscopy

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Increasing evidence suggests that a full understanding of biomolecular function and disease requires in-situ approaches that probe molecular structural and dynamics in a native setting. NMR is a non-invasive method that has made significant progress to study biomolecular systems in a native-like environment including bacterial, fungal or human cells. In our contribution, we will introduce novel solid-state NMR (ssNMR) techniques to study complex biomolecular systems in a bacterial[1,2] and human cell[3-5] setting. These methods maximize spectral resolution and sensitivity and are geared towards elucidating complex molecular systems including microtubular protein complexes[5] as well as deciphering the dynamic landscape of proteins inside bacterial and human cells and cell organelles. Examples of such applications will be shown.

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Application of electron paramagnetic resonance (EPR) spectroscopy in dermatology

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The redox status and oxidative stress investigations can be performed *in vitro, ex vivo* and *in vivo* using EPR spectroscopy [1]. This permits evaluating antioxidant supplements and formulations but also studying the influence of oxidative stress, which could be induced by irradiation, lifestyle [2], cold plasma application or pollution. For example, the formation of radicals caused by the different spectral regions (UVC, UVB, UVA, VIS, NIR) can be investigated using the spin probe PCA [3, 4] (quantification) and spin traps (characterization) [4].

PCA is also well suited for drug labeling. If a spin labeled drug is loaded to nanocarriers the localization of the drug within the carrier, the penetration efficiency into the skin and a possible release can be studied in intact and barrier disrupted skin [5, 6].

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Advanced biophysical visualization methods for nanomedicine and theranostics

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In recent years, my group has specialized in developing time-resolved fluorescence spectroscopy techniques into advanced spectromicroscopic methods for use in nanomedicine. We developed a fast, precise, fitting-free cluster-based fluorescence lifetime imaging method (Cluster-FLIM) that provides images with enhanced information, contrast, and spatial resolution at short exposure times and low fluorophore concentrations [1,2]. Thereby, Cluster-FLIM increases the applicability of FLIM in high content analysis of target molecules in drug development and treatment monitoring. We focus on nanocarrier characterization and non-invasive topical drug delivery visualization [2-5], and nanocarrier/drug interactome determination in living cells, 3D tissue models, or ex-vivo tissue [1,4]. Molecular rotors are used as sensitive fluorescent probes for biomolecular interaction and in theranostic approaches [1,6]. As nanocarriers may also induce harmful oxidative stress to the cells, we developed a highly sensitive fluorescence lifetime-based reactive oxygen species (ROS) detection assay, FLIM-ROX [7]. Currently, we develop green lipid nanocarriers for effectively transporting natural compounds with antibacterial and anti-inflammatory activity [8] and use FLIM-ROX for treatment monitoring and a single molecule tracking-free method, Diffusivity Analysis of NAnoscopic Ensembles (DANAE) [9], for diffusivity measurements.

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Multiscale Fluorescence Imaging of the Liver - from the Mesoscale to the Nanoscale

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The liver is the largest organ in most mammalian bodies and is composed of a complex macroscopic and microscopic architecture that supports its indispensable function to maintain physiological homeostasis. Optical imaging of the human liver is particularly challenging because of the need to cover length scales across 7 orders of magnitude (from the centimeter scale to the nanometer scale) in order to fully assess the ultrastructure of the entire organ [1]. This task becomes even more challenging the deeper within the organ one hopes to image, because of the strong absorption and scattering of visible light by the liver. I will demonstrate how optical imaging methods utilizing highly specific fluorescent labels as well as label-free optical methods can seamlessly cover this entire size range in excised, fixed human and rodent liver tissue. I will present and explain the successful use of optical projection tomography and multi-photon light-sheet fluorescence microscopy to derive information about the liver architecture on the millimeter scale [1]. The intermediate size range is covered using label-free structural and chemically sensitive methods, such as second harmonic generation and coherent anti-Stokes Raman scattering microscopy [2]. Extended field-of-view super-resolution optical microscopy methods such as waveguide-based single molecule localization microscopy [3] and fiber-optics based structured illumination microscopy allow us to extend the resolution to the nanoscale [4, 5], enabling us to ultimately image individual liver sinusoidal endothelial cells and their fenestrations by super-resolution [6].

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High-end microscopy of peroxisomes

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Peroxisomes are ubiquitous cell organelles with critical health-related functions in lipid and reactive oxygen species metabolism. With a diameter of approximately 100 nm [1], the size and morphological features of peroxisomes are below the diffraction limit of light, which makes them attractive for super-resolution microscopy (SRM) [1, 2]. Moreover, the biogenesis of peroxisomes and the mechanism of pathology development in patients and models of peroxisome deficiency are poorly understood. We contributed to the study of peroxisomes by SRM [1, 2] and to the development of more specific tool for the study of intraperoxisomal metabolites in excitable cells by FRET [3, 4]. We will further present SRM data on cardiac peroxisomes in a novel mouse model of peroxisome dysfunction (unpublished). More generally, the presentation will emphasize the need to advance the study of peroxisomes by SRM.

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Abstracts of Posters

(in alphabetical order)

Next Generation Cell-Penetrating Peptide Additives

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Inefficient cellular uptake significantly limits the application of therapeutic biomacromolecules. For example, antibody drug therapy is restricted to targeting and blocking membrane-bound protein, leaving a plethora of intracellular targets untouched. Hence, we ask: “How do we use chemistry to cross biological borders?” Conjugating cell-penetrating peptides (CPP) onto proteins of interest enables cellular uptake through either endosomal uptake of via direct membrane transduction, which is the preferred delivery route.[1] Unfortunately, this entry is only observed cells are treated with high concentrations of CPP-protein conjugates.[2-4] This requirement is detrimental to the cell’s health and will prove costly for future applications. With the aim to improve safety and efficiency, we looked into the mechanism of CPPs to design a novel strategy for direct cytosolic delivery.

We answered this problem by designing CPP-additives. These contain cell-surface reactive moiety and a decaarginine motif, and allow direct cytosolic uptake of CPP-protein conjugates through the cell membrane at a lower treatment concentration.[5] We greatly improved the system by adding a hydrophobic motif consisting of cell membrane interacting amino acids. Hydrophobic CPP-additives show enhanced cell membrane interaction and creates areas of loose membrane lipid packing, through which the CPP-proteins enter quite efficiently, without compromising cell viability. This presentation will discuss the development of the strategy and the most current improvements on the CPP-additives.

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In Situ ATR FTIR Spectroscopy as a Benchmark for the Catalytic Reactions of Complex IV

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Complex IV of the mitochondrial respiratory chain, or cytochrome *c* oxidase, contributes largely to the proton motive force necessary for ATP synthesis. It exploits the energy gap between the redox couples O_2/H_2O and cytc^{2+}/cytc^{3+}, pumping protons against the membrane gradient while using molecular oxygen as a substrate [1]. In contrast to other mitochondrial complexes, Complex IV does not produce reactive oxygen species (ROS). Rather, a second mechanism of respiratory control has been proposed, in that Complex IV can decrease the formation of ROS thanks to an allosteric inhibition mechanism involving ATP [2].

The study of this enzyme is critical for physiology and drug development, and consequently Complex IV has been extensively analyzed by several different techniques in the last decades.

With our work, we present a dedicated gas titration protocol for the analysis of mid-IR difference spectra of bacterial cytochrome *c* oxidase. We can easily prepare the fully-reduced or mixed-valence states of the enzyme (4 e^-- or 2 e^--reduced) and quickly induce their oxidation by O_2, producing relevant marker bands that can be compared between different reactions. The reactions can be induced without the use of light or inhibiting ligands like carbon monoxide (CO), making our setup ideal for benchmarking different environmental conditions or mutants. Here we present the effects of different lipid reconstitutions and ligand binding procedures on cytochrome *c* oxidase from *Rhodobacter sphaeroides*.

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Imaging the Uptake and Effects of Microplastics on Organisms by Coherent Raman Scattering Microscopy

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In order to determine the effects that the uptake of microplastics has on various organisms, novel means of detecting and imaging microplastics inside cells and organisms are needed. In the best case, such methods should provide entirely label-free contrast, while still being chemically specific. In optical microscopy, this is best realized by utilizing Raman scattering, which is the inelastic scattering of light by molecular bonds. While spontaneous Raman scattering is too slow for most imaging applications, coherent Raman scattering (CRS) can be employed at imaging speeds up to video-rate. We have developed and employed a fully customizable CRS microscope, which allows us to visualize the structure of organic and biological samples in the same imaging process as the identification and imaging of microplastics in these samples. This system allows us to analyze a wide range of effects of the uptake of microplastics, e.g. by plant seedling up to monitoring fitness parameters, such as lipid concentration in C. elegans nematodes. A variety of techniques are combined in one setup. These techniques include coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) using optical parametric oscillators to probe different Raman resonances, as well as second harmonic generation (SHG) to probe tissue structure. This gives us the opportunity to use a wide range of analysis techniques to collect information with biological and medical relevance. These methods include volumetric imaging and hyperspectral analysis to produce quantitative data about the composition of the examined samples. In this contribution we will provide detailed information about the setup and recent improvements, and demonstrate its wide range of applications with particular emphasis on analyzing the effects of microplastics uptake by organisms.

Improving cell specificity of cell-penetrating peptide additives

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Conjugating cell-penetrating peptides (CPP) onto proteins of interest enables cellular uptake through either endosomal uptake of via direct membrane transduction, which is the preferred delivery route.[1] Unfortunately, this entry is only observed cells are treated with high concentrations of CPP-protein conjugates.[2-4] This requirement is detrimental to the cell's health and will prove costly for future applications. With the aim to improve safety and efficiency, we looked into the mechanism of CPPs to design a novel strategy for direct cytosolic delivery.

We designed CPP-additives that contains cell-surface reactive moiety and a decaarginine motif, and allow direct cytosolic uptake of CPP-protein conjugates through the cell membrane at a lower treatment concentration.[5] We greatly improved the efficiency of the system by adding a simple hydrophobic motif consisting of cell membrane interacting amino acids. Hydrophobic CPP-additives show enhanced cell membrane interaction and creates areas of loose membrane lipid packing, through which the CPP-proteins enter quite efficiently, without compromising cell viability. In addition, we have also shown that N-terminal modification of the decaarginine CPP-additives by using phenylboronic acids can improve cytosolic delivery of proteins. Phenylboronic acids are well known for glycan recognition on the cell surface, which allows a better attachment of the additives to the cell surface.[6-7] Also, this approach could also be used to gain some selectivity in hypersialilation processes present on cancer.[8] This presentation will discuss the development of the strategy and the most current improvements on the CPP-additives.

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Tracking water oxidation through time-resolved FTIR Spectroscopy

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In the light-driven reaction cycle of Photosystem II (PSII), two 'substrate' water molecules are oxidized resulting in the release of O_2 at the oxygen-evolving complex (OEC), which consists of a Mn_4Ca-oxo cluster and its water-protein environment. Driven by a sequence of light flashes, the OEC cycles through its five S-states, alternating the release of electrons and protons.

Infrared (IR) spectroscopy offers unique insight into these processes, as protonation states and even slight structural or functionally crucial H-bond networks can be resolved. However, experiments have restricted themselves to either spectrally extensive Fourier Transform IR (FTIR) investigations of semi-stable intermediates without time-resolution[1] or recent time-resolved studies focusing on select bands [2,3].

To investigate events of the PSII photocycle, we heavily modified the sample compartments of two commercial FTIR spectrometers to push the experiments beyond previous limits. Custom sample changers allow for efficient acquisition and processing of thousands of excitation sequences.

In a step-scan experiment, more than 230,000 excitation cycles of dark-adapted PSII were recorded to resolve processes from 50 µs to 130 ms over a range of 1300-1800 cm^{-1}, for all S-state transitions. Alongside quantum chemical simulations, analysis of spectral features associated with O_2 evolution identifies a transient carboxylate deprotonation preceding the formation of an oxygen radical at the OEC [4]. In another series of experiments pushing rapid-scan time resolution below 10 ms allows for the investigation of PSII systems with slowed kinetics (mutations, water analogues, etc.).

Besides informing artificial water splitting catalyst design, our results improve the understanding of basic biological mechanisms, e.g., in photosynthetic proteins or proton pumps.

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The dHis-Cu^{2+}(NTA) Motif: Site Specificity of Cu^{2+}(NTA)-Binding and Evaluation of Pulsed Dipolar EPR Data Involving this Motif

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Enzymatic activity of proteins and other biomolecules is closely linked to the structure and its structural rearrangement. While pulsed dipolar EPR spectroscopy (PDS) in combination with site-directed spin labeling is a widely applied tool to investigate such structural rearrangements, the conformational flexibility of spin labels can obstruct the detection of conformational changes within a biomolecule.[1] A way to circumvent this is the use of the bipodal double-histidine motif in complex with Cu^{2+}, where the labeling site is located closely to the protein backbone and rotational freedom of the label is restricted.[2] However, due to the stiffness of the label, the spectral width and large g-anisotropy, orientational selectivity can make the analysis of the PDS data more involved.[3] Here, we provide guidelines for site-selective labeling using the dHis-Cu^{2+}(NTA) motif and compare RIDME (relaxation-induced dipolar modulation enhancement)[4] and PELDOR (pulsed electron-electron double resonance)[5] with respect to their sensitivity and susceptibility to orientational selectivity. Using the Yersinia outer protein O (YopO) as a model, we could demonstrate that the labeled helix backbone shows a unimodal distance distribution, hence suggesting that dHis-Cu^{2+}(NTA) labeled α-helix of YopO adopts only one conformation. This contrasts with previous studies with other labels, which suggested two distinct helix conformations, straight and bent.[6] Our study shows that great care must be taken when interpreting multimodal distance distributions and the dHis-Cu^{2+}(NTA) label is well-suited to resolve small structural deviations in proteins.

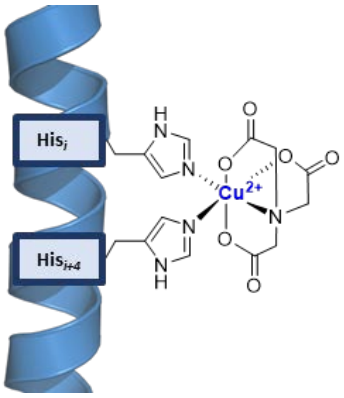

Figure 1: dHis-Cu^{2+}(NTA) motif located on an α-helix.

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Chiral structured illumination microscopy for simultaneous imaging of chiral and achiral domains

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The ability to fast image chiral domains at sub-diffraction limited resolution is valuable for understanding the spatial distribution of chiral species and may find applications in drug design, material sciences, and biomedical imaging. Previously, we proposed a new chiral domain imaging modality, named "Chiral Structured-illumination Microscopy (Chiral SIM)". This method is based on optical chirality engineering, fluorescence-detected circular dichroism, and structured illumination microscopy. In chiral SIM, the optical chirality of the illumination is structured and the circular dichroism-dependent fluorescence from chromophores near molecular chiral domains is detected. With image reconstruction, the spatial distribution of chiral domains with sub-diffraction limited resolution can be obtained [1]. Here, we show that chiral SIM can be easily extended to "Double SIM", which enables simultaneous imaging of achiral and chiral domains at a sub-wavelength resolution [2]. In double SIM, the illumination field is spatially structured both in the intensity and optical chirality so that moiré effects can be concurrently generated on the achiral and chiral fluorescent domains of a sample. This allows for down-modulating the high spatial frequency of both domains at the same time and thus provides subwavelength details after image reconstruction. We introduce the working principle and theoretically demonstrate the feasibility of these methods.

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Non-uniform holographic grating as a virtual noninvasive probe

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Optical diagnostic methods and techniques have proven to be indispensable for the studies of many of biological objects and systems. Advances in optical techniques have enabled noninvasive, *in situ* characterization of different processes and functional states of the body. The use of light and optics provides many advantages such as a multi-dimensional data collection on different scales. Development of nonlinear optical microscopy and optical coherence tomography has enabled depth-resolved, high-resolution imaging of important organs and features.

Nonlinear Interaction of light waves in a medium appeared to be an efficient tool for manipulation of laser beams and detection of parameters of a medium. Two- and four-wave mixing techniques in nonlinear media are widely used for amplification of laser beams and optical images, phase conjugation, and in a variety of optical sensors, etc.

A model liquid crystal (LC) based system is presented allowing for parametric studies of the processes of formation of a non-uniform phase grating profile and investigations of its influence on coherent waves coupling. Local light-stimulated modulation of the LC order parameter provides large optical nonlinearities and extremely fast as for the LC system recording times opening new possibilities for the volume grating recording. Fine tuning of the phase shift between an interference pattern and a refractive index grating should result in a dynamic holographic non-locality that could be used for imaging of hidden inhomogeneities.

Probing Protein Dynamics using Quantum Cascade Laser based Surface Enhanced Infrared Absorption Spectroscopy

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Understanding protein dynamics and their underlying molecular mechanisms is a crucial part for developing a detailed description and explanation of protein function. Infrared spectroscopy as a label-free technique is perfectly suited to address related questions by directly and non-invasively probing molecular vibrations. While established Fourier-transform infrared methods either struggle with low time resolution or high demands with respect to sample stability, quantum cascade laser (QCL) based approaches were already demonstrated to be capable of overcoming those limitations [1]. Using surface enhanced infrared absorption spectroscopy (SEIRAS) - a technique in which the sample is being probed in attenuated total reflection configuration - the experimentalist has free access to the sample for adjusting important conditions like pH. Additionally, a variety of triggers for initiating reactions can be employed. In this work we present a combination of QCL based IR spectroscopy and SEIRAS which has great potential for exciting future experiments.

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EPR spectroscopy as read-out system of redox homeostasis in blood plasma with UVA irradiation as a stressor

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Oxidative stress as a disease driver reinforces the trend towards supplementation with antioxidants. At physiological doses, antioxidants positively influence the redox status; higher concentrations may have pro-oxidative effects.
Using in-situ-irradiation as stressor and electron paramagnetic resonance (EPR) spectroscopy as readout system for formed radicals, a stress response assessment method was developed and validated in a double-blind placebo-controlled in vivo cross-over pilot study in blood plasma samples before and after vitamin C supplementation. Reference measurements were performed for oxidative stress indication. Plasma samples displayed two slopes (m1, m2) for radical production, whereby m1 represented the amount of antioxidants and proteins, m2 only the protein content. These two slopes in combination with the intervening transition area (T) were used to calculate the oxidative stress coping capacity (OSC), which correlated positively with vitamin C concentration in blood plasma. Furthermore, a selective radical quenching mechanism for vitamin C was observed: the proportion of reactive oxygen species in the plasma samples was degraded in dependence to the vitamin C concentration ingested; the proportion of lipid oxygen species remained stable. OSC may represent a sensitive method to detect treatment effects on the redox status in vivo in future validation and treatment studies.

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Advancements in Time-Resolved Single-Frequency Infrared Spectroscopy on Photosystem I and II

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In plants and cyanobacteria, the large protein complex Photosystem II (PSII) catalyses light-driven oxidative water splitting, yielding O_2, protons and electrons. Further down the photosynthetic chain, Photosystem I (PSI) catalyses the electron transfer (ET) from plastocyanin to ferredoxin, leading to the formation of NADPH.
When using laser flashes to excite PSII or PSI, variations in protein-internal H-bond networks and electric fields as well as metal-ion oxidation can be investigated by infrared (IR) difference spectroscopy. Employing tunable quantum cascade lasers (QCL), we have developed a time-resolved single-frequency (TRSF) IR experiment (Fig. 1) with automated sample exchange that facilitates tracking of ET and proton transfer (PT) dynamics at high temporal resolution (signal half-rise time ~ 36 ns).[1]
PSII from spinach was investigated in the mid-IR regime at several pH values in H_2O and D_2O. By reproducing results i.a. by photothermal spectroscopy[2] we show that the ET and PT events of the S-state cycle are 'sensed' by specific carboxylate and amide vibrations.
By stepwise tuning through the QCL, we were able to reproduce Fourier-Transform-IR (FTIR) difference spectra for three PSI variants in a short amount of time and excellent signal to noise (S/N).

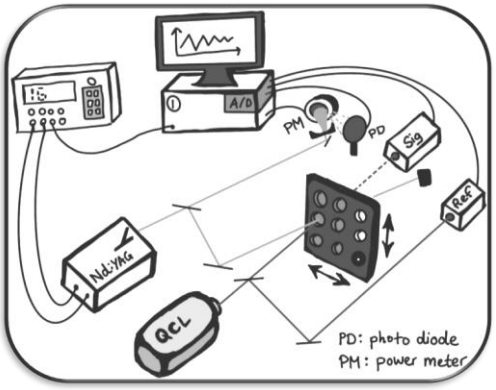

Fig. 1: Scheme of the TRSF-IR Setup

For PSII we recently succeeded in acquiring time-resolved data sets covering 1310 to 1750 cm^{-1} in 2cm^{-1} steps. We conclude for PSII and likely other systems with critical S/N, the QCL experiment is superior to classical step-scan FTIR regarding the time needed for the collection of data with high spatial and spectral resolution.
By employing light-induced delivery systems or a flow-cell mixing system, TRSF-IR may also be used for systems other than light-driven proteins, e.g. for the analysis of specific pharmacokinetical steps. By monitoring both kinetics and spectral changes upon a trigger event (pH, light, temperature), the accumulation of (side-)products may become observable during e.g. *in vitro* drug delivery.

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High-speed TIRF and 3D super-resolution structured illumination microscope with large field of view based on fiber optic components

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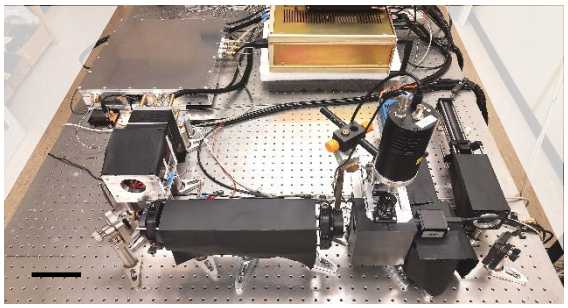
Super-resolved structured illumination microscopy (SR-SIM) is among the most flexible, fastest and least perturbing fluorescence microscopy techniques capable of surpassing the optical diffraction limit. Current custom-built instruments are easily able to deliver two-fold resolution enhancement at video-rate frame rates, but the cost of the instruments is still relatively high and the physical size of the instruments is still prohibitively large. We have developed a compact, cost-efficient and high-speed fiber-based capable of 2D-, grazing-incidence-, TIRF and 3D-SIM to overcome these limitations. The fiber-based illumination path allows for multi-color imaging with a field-of-view (FOV) of up to 150x150µm². The setup can be extended with a real-time--reconstruction to have the look-and-feel of a wide-field microscope.

The setup consists of a fiber-coupled laser combiner with 405nm, 491nm, 561nm and 639nm laser sources, a fiber-switch to select the desired illumination angle with integrated phase shifters and a hexagonal fiber collimator with a lens telescope that projects the beams into the back-focal plane of the objective lens. All modules of the illumination path are home-built to achieve highest stability and a compact size. The rotation of the illumination angle is performed by selecting the corresponding pair of broadband, polarization-maintaining fibers by two galvo-mirrors in the fiber-switch. The phase shifts of the zero order beam and one of the two first order beams are performed by home-built, MEMS-mirror based phase-shifters that modulates the optical path length. The phase shifters and galvo-mirrors allow transition times between illumination patterns of less than 1ms and are achromatic. The hexagonal collimator allows adoption of the Gustafsson-SIM approach while being able to adjust the pattern spacing in the sample continuously, within a transition time of 100ms.



Photograph of the setup (scale bar 10cm)

The capability of the setup is demonstrated by multicolor imaging of fixed liver endothelial cells (LSECs) with TIRF- and 2D-SIM with a FOV of up to 150x150µm². The resolution limit is pushed to 85nm by TIRF-SIM which allows to analyze the number and shape of fenestrations of LSECs. The next steps will be software development to reconstruct 3D-SIM datasets and a combination with real-time-reconstruction algorithms. This will allow the analysis of living cells with TIRF- and 3D-SIM.

Actin’ in Cell Division

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Cell division is essential for life. However, many mechanisms that control the striking changes in cell morphology during mitosis are still enigmatic. Using confocal live-cell imaging we discover two groups of dynamic actin filaments that are required for proper mitosis by maintaining genomic integrity through individual mechanisms.

On one hand, we identify Arp2/3-dependent actin filament assembly spatially defined at centrosomes contemporaneously with spindle microtubules forming during prometaphase. Pharmacological Arp2/3 complex inhibition and overexpression of the Arp2/3 inhibitory protein Arpin results in decreased spindle actin, impaired spindle assembly and mitotic defects.

On the other hand, we visualize the transient formation of nuclear actin filaments during mitotic exit. These structures assemble in daughter cell nuclei and undergo dynamic reorganization to promote nuclear protrusions and volume expansion throughout the early G1 phase of the cell cycle. Specific inhibition of nuclear actin assembly impaired nuclear expansion and chromatin decondensation. Biochemical screening for mitotic nuclear F-actin interactors identified the actin-disassembling factor cofilin-1. Optogenetic regulation of cofilin-1 revealed its critical role for control-ling timing and turnover of F-actin assembly inside daughter cell nuclei.

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Non-Invasive Blood Glucose Measuring Device: from Lab to Bed Side

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Non-invasive glucose measurements in human skin in vivo is one of the biggest challenges in the research on diabetes. Currently many research approaches in this field are investigated but there is no reliable product on the market yet [1].

Our approach is based on the availability of tuneable Quantum Cascade Lasers (QCL) as powerful Mid-Infrared laser sources. The infrared fingerprint absorption bands of glucose in the epidermis (and other skin components) in the wavelength range 8-11 µm can be selectively excited by the laser; this selectivity ensures molecular specificity. Excitation and subsequent thermal relaxation of glucose leads to deposition of a tiny amount of heat in skin; the readout of this thermal signal is measured optically, with the help of a prism, resulting in a Photo-Thermal-Deflectometry signal (PTD) [2]. The PTD technology is sensitive and reliable, and a device composed of mostly off-the-shelf components has been already certified as medical product [3].

The bumpy road from a table-top laboratory spectrometer to a miniaturized hand-held device will be briefly presented here. Although the first prototypes of QCLs appeared at the end of the 20th century, only recently laser companies started to shrink the size of their products. As the QCL is the central unit of our devices we are working on the development of a miniaturized QCL-array of single emitters together with the german manufacturer Nanoplus Nanosystems and Technologies GmbH.

In collaboration with RWTH Aachen University we are also conducting multi-physics numerical simulations in order to better understand the skin-prism interaction and therefore optimize the measuring hardware and software parameters.

Altogether the obtained results led to the development of a hand-held prototype and bring us closer to the market release of a non-invasive glucose measurement device for the bed side.

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Protein Conformational and Protonation Dynamics Monitored by Single-Shot IR Spectroscopy

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Abstract:
Time-resolved IR-spectroscopy is a label-free technique to study structure-function relationships in proteins. The established method of step-scan FT-IR spectroscopy yields spectrally broad information (>3000cm^{-1}) with sufficiently high time resolution (up to ns) but strictly requires reversible processes and stable samples making it only suited for a limited number of systems, such as bacteriorhodopsin. [1] Although setups based on tunable quantum cascade lasers (QCL) are capable of collecting kinetic information with ns time resolution by a single acquisition, the spectral information is limited to a single wavelength [2]. Contrary, QCL-based dual comb spectroscopy (DCS) allows for a simultaneous broadband (~60cm^{-1}) observation of reaction kinetics with µs time resolution [3,4].
In this study we used quantum cascade laser (QCL) based dual-comb spectroscopy to study protein conformational and protonation dynamics of the well characterized membrane protein bacteriorhodopsin. We provide a comparison between a commercially available dual-comb spectrometer and our homebuilt tunable QCL-based spectrometer to showcase the possibility of probing non-repetitive protein dynamics. Our results show that with the use of QCLs it is possible to observe transient conformational changes as well as protonation events of single amino acids within a protein, without any acquisition averaging necessary. Thus, we expect QCL-based approaches to be powerful tools to open the field of mid-IR spectroscopy to the of study reaction mechanisms of irreversible (bio)-chemical transformations.[5]

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Three-dimensional optical projection tomography of biomedical tissues

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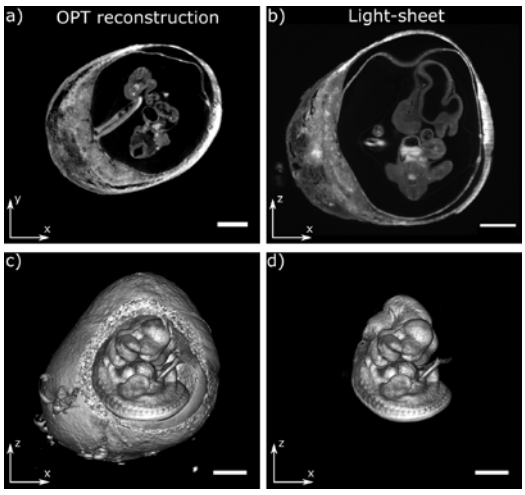
In the field of optical microscopy, the size range of biological and medical samples of interest varies from single biomolecules (nanometre scale) to whole animal organs (centimetre scale). Since this size range spans across 7 orders of magnitude, different optical tools are needed to analyze such samples across these scales. The mesoscopic size range is concerned with the larger structure of samples up to several centimetres. For such large samples, the penetration depth of optical microscopy is limited by the interaction of light with tissue causing absorption and scattering. The amount of scattering depends on the thickness and the transparency of the sample. The larger the sample, the lower is the best achievable resolution. With the use of optical projection tomography (OPT) it is possible to image and reconstruct the internal three-dimensional structure of such large samples. For example, entire intact murine organs are visualized without the need for physically cutting the tissue. The sample must, however, be cleared before imaging to improve the penetration depth of the visible light and fully resolve the internal structure. We are currently working on improving the whole mount staining process using indirect antibody labelling for different types of biological samples, i.e. mouse organs, mouse embryos and human nasal polyps. Optical clearing is performed using the organic solvents benzoic acid and benzyl benzoate (BABB) and we are characterizing the effect of pre-bleaching the tissue with H_20_2. The OPT setup is combined with a mesoscopic light-sheet microscope to compare the resulting reconstructed images and the resolution of both techniques based on the same sample. OPT is used to characterize the internal structure, i.e. the three-dimensional distribution of blood vessels and calculating i.e. the microscopic vessel density. Our current status of improving the imaging conditions, sample preparation and the optical setup will be detailed.



Autofluorescence measurement of a E9.5 mouse embryo yolk sac. a) - b) Cross-sections of the sample created with the OPT and light-sheet. c) – d) The 3D structure of the embryo. Scale bar 1 mm.

3D short-wave infrared (SWIR) imaging – Developing an optical projection tomography (OPT) setup

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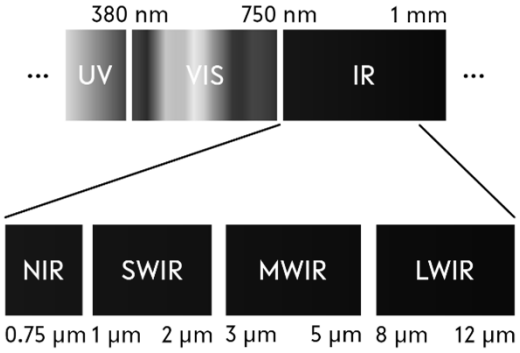
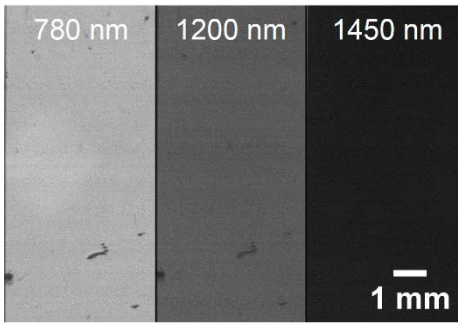
In order to resolve the internal structure of extended biological samples, i.e. entire organisms, organs, or tissue sections, by optical microscopy, these samples require optical clearing in order to become optically transparent. Optical clearing typically involves an extensive and harsh chemical treatment process, where samples are first fixed by chemical crosslinking, lipids are removed by transfer from aqueous buffers to 100% alcohol, and finally chromophores are removed or bleached. Obviously, this harmful process cannot be extended to living specimen. Due to reduced scattering and absorption, light in the short-wave infrared (SWIR) region, however, can penetrate much deeper into biological samples than visible light. Often penetration depths up to many millimeters are possible – without the need for optical clearing. We are currently developing an optical projection tomography (OPT) setup capable of resolving the internal structure of optical samples down to the sub-100 µm scale that utilizes a high pixel-count camera sensitive between 400–1700 nm. This will allow us to utilize multiple contrast mechanisms in the infrared spectral region, i.e. absorption and fluorescence of SWIR-active fluorophores to image the water and lipid distribution in these specimen as well as specifically labeled molecular structures. Our current status with regard to these developments will be detailed and explained, and a short outlook to future applications will be provided.





Water absorption for different wavelengths. Water has a prominent absorption feature near 1450 nm. Wavelength specific absorption features can be used to distinguish different materials.

Intracellular visualization of SARS-CoV-2 RNA by super-resolution microscopy

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Since the start of the COVID-19 pandemic, caused by the severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), a tremendous amount of research has been published on this subject with the aim to facilitate the development of efficient therapeutic drugs. Over the years, direct visualization of viral RNA in infected cells, a critical step to analyze viral replication, has been established and applied to SARS-CoV-2. [1]

The surface of the SARS-CoV-2 virus capsid is covered in spike-(S)-proteins, while the capsid itself contains the positive-sense single-stranded RNA molecule. This RNA molecule is used as a template for virus replication in the host cell. The SARS-CoV-2 RNA has been used by Rensen et al. 2022 [1] as a target for single-molecule inexpensive FISH (smiFISH), which allows for highly specific staining of viral particles. As shown in Rensen et al. their smiFISH approach is suited for immunofluorescence and electron microscopy imaging methods. However, since sample preparation especially for electron microscopy is complex and time consuming, we optimized the smiFISH protocols for super-resolution light microscopy e.g. Lattice-SIM and dSTORM. This provides us with an efficient way of visualizing SARS-CoV-2 RNA at a high resolution. Moreover, we were able to combine the smiFISH approach with immunofluorescence methods, which allows for costainings with additional targets besides the virus itself. Thus, enabling detailed studies of viral infection, replication and egress.

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Perfusion-Induced FTIR Difference Spectroscopy on Human ABC Transporters

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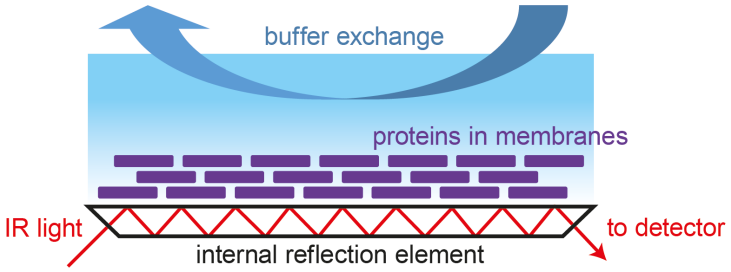
Attenuated Total Reflection (ATR) FTIR spectroscopy is a powerful tool for the non-invasive investigation of the mechanism of membrane proteins. The immobilization on the surface of the internal reflection element enables for the exchange of the buffer solution above the adsorbed sample film by perfusion. Detected difference signals can then be assigned to originate from the stimulus by the change of conditions. Here, we developed a modular attachment for a diamond ATR cell to produce a flow cell with 500 microliter volume, which enables for the reproducible and controlled exchange of buffer solution above membrane stacks by a syringe pump. Accessible stimuli are a change in pH and ion composition or addition of substrates and inhibitors.



ATP binding cassette (ABC) transporters are an important class of membrane proteins which use the hydrolysis of ATP for the active transport of substrates across membranes. In humans 48 different ABC transporters can be found. Many of them are medically relevant, for example ABCB1 (p-glycoprotein) is associated with multidrug resistance in cancer therapy due to its exporter function. Others are involved in diseases like diabetes or cystic fibrosis.

The human mitochondrial transporter ABCB10 is involved in heme biosynthesis and protects cells from oxidative stress. The transporter was recombinantly expressed in *E. coli* and membrane fragments were generated. Using perfusion-induced ATR FTIR difference spectroscopy the response of ABCB10 to the addition of ATP was characterized. The hydrolysis of ATP and accompanying changes in α-helical secondary structures were observed. These results show that ABCB10 undergoes structural changes typical for ABC transporters. Moreover, the response of ABCB10 to its potential transported substrate Zn(II) mesoporphyrin is analyzed. In the future, we will investigate membrane preparations from human cell lines to gain further insight to the mechanism of the human ABC transporters.

Following conformational changes in *Lbu*Cas13a from apo to ternary cr- and target RNA bound state with EPR spectroscopic distance measurements

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The type VI CRISPR effector protein Cas13a is an RNA nuclease, catalyzing two RNA cleavage events: First, the maturation of pre-crRNA and second, the cleavage of target RNA, activating a collateral cleavage.[1] Both, target and collateral cleavage make Cas13a a powerful tool for medicinal applications ranging from the detection of pathogens as SARS-CoV-2 *in vitro* and in mice[2], to the treatment of influenza, SARS-CoV-2[3], and glioma intracranial tumors in mice.[4] Although all four structures of Cas13a on route from apo[5], via the bound pre-crRNA[6] ($Cas13a^{precr}$), the crRNA[8] ($Cas13a^{cr}$), and the crRNA-target RNA[7] ($Cas13a^{cr+target}$) complex are known, they origin from proteins of different organisms and contain different extends of truncation. To identify and follow conformational changes on a molecular level, structures of Cas13a in all states from one organism are needed. We aim to resolve static structures and follow conformational changes by site directed spin labelling in combination with Pulsed Electron-Electron Double Resonance (PELDOR)[8]. To this end, we labeled *Leptotrichia buccalis* Cas13a with pairs of nitroxide spin labels at different positions and tested their influence on the functionality. All active constructs were subjected to PELDOR experiments. We compared the resulting distance distributions with and without RNA to *in silico* generated distributions, calculated with the software mtsslWizard[9], based on the experimental RNA bound structures and the AlphaFold2[10] (AF2) structure as input. We show, that the structures of $Cas13a^{cr+target}$ and the $Cas13a^{cr}$ are very similar to the experimental structures. The unknown $Cas13a^{precr}$ conformation resembles $Cas13a^{cr}$ and the unknown apo state shows a wide open, flexible domain conformation, which is not well described by AF2.

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Skin safety of 233 nm Far-UVC irradiation in *ex vivo* human skin and healthy humans

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UVC irradiation is a promising method for eradicating microbes without development of resistance by triggering photolesions in DNA or RNA of microorganisms. Therefore, UVC irradiation at 254 nm is increasingly used for indoor air and surface decontamination. However, the use of UVC radiation in public areas is prohibited due to its carcinogenic effect on skin and mucosa and the risk of cataract formation in the eye. Far-UVC light, typically defined as 200–235 nm, is a promising alternative: It exhibits antimicrobial properties similar to 254 nm light, but without inducing damage to the human skin or eye due to the reduced penetration depth. A recently developed 233 nm far-UVC LED source has been shown to be strongly absorbed by the stratum corneum, protecting the keratinocytes in the viable epidermis, but eradicating pathogens present on the skin surface [1], [2]. Recently, *in vitro* studies have reported that irradiation with 60 mJ/cm² at 233 nm is sufficient to eradicate a broad spectrum of microbial pathogens, including MRSA (Methicillin-resistant *Staphylococcus aureus*) and fungal spores [3]. Hence, far-UVC irradiation can be a potential tool for *in vivo* skin antisepsis and decontamination of public locations in presence of humans.

Here, we report the results of the skin safety evaluation of 233 nm far-UVC irradiation on *ex vivo* human skin and for the first time in healthy humans *in vivo*. Comparisons were made with a broadband UVB lamp used as positive control. The irradiation with 60 mJ/cm² at 233 nm induced almost no premutagenic UV-associated DNA lesions. Compared to 1/10 minimum erythema dose (MED) *ex vivo* and 1 MED for UVB *in vivo*, a dose at which only detectable skin redness occurs, far-UVC irradiation does not induce any inflammatory response and damage was minimal and only superficial.

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