# Dear Colleagues,

We would like to welcome you to our Translational Bioimaging Symposium 2024. This symposium brings together established scientists, younger investigators and postdocs to discuss the state-of-the-art in the development and use of genetic modifications (e.g. genetic code expansion), novel chemical reactions (e.g. bioorthogonal click chemistry), fluorescent probes and microscopy for future biomedical treatment or analysis.

The meeting is organised by the local Rudolf Virchow Center, an interdisciplinary research center focusing on visualization of elementary life processes from the subnano to the macro scale. As a central institution of the University of Würzburg, the RVZ is currently home to 14 research groups with interdisciplinary and translational research focus, and to about 100 scientists who investigate the molecular causes of health and disease. Our scientists have pioneered and established methods and technology concerning structural biology, (super-resolution) fluorescence microscopy, proteomics and mass spectrometry.

| | |
|---|---|
| Scientific organisers: | Gerti Beliu and Katrin Heinze |
| Administrative organisers: | Felix Knote, Line Jung, Jana Endres and Ole Riemann |
| Official Website: | www.uni-wuerzburg.de/rvz/bioimaging2024 |
| Twitter: | @rvz_wuerzburg #Bioimaging2024 |
| Contact E-mail: | rvz-bioimaging2024@uni-wuerzburg.de |

## Location

Rudolf Virchow Center
University of Würzburg
Josef-Schneider-Str. 2, Haus D15
97080 Würzburg, Germany

## Coats and bags

An unattended coat rack can be found at the back of lecture hall with some space for luggage and bags.

## Name tags

Please pick up your name tag at the registration desk. We kindly ask you to wear it throughout the meeting. Please ask the registration desk if you need a new name tag.

## Photos

The Rudolf Virchow Center will be taking photos during this symposium. These images may be used by the Rudolf Virchow Center for press releases, printed publicity, news and published on the Rudolf Virchow Center Twitter Page. If you prefer not to be photographed, please speak to the organisers.

## Conference Dinner

The conference dinner will be on the 17th June, from 18:30 - 22:30. We will stay at the Hofkeller at Residence Wuerzburg, starting at the fountain in the front of Residence (parking lot) with a guided tour, wine tasting and visit to the wine cellar. The bus transfer will leave at 18:15 in front of the Rudolf Virchow Center. Please note that a pre-registration was required (conference registration), as the dinner was charged with 71€ extra.
Address: Residenz Würzburg, Residenzplatz 2, 97070 Würzburg (tram no 1 & 5, stop Barbarossaplatz, walk 10 minutes )

## Hotel

The nearby GHOTEL hotel Würzburg ist located at:
Schweinfurter Straße 3
97080 Würzburg
Telefon: +49 931 35962 – 0
Email: wuerzburg@ghotel.de

## Certificate of attendance

Please inform the registration desk if you need a certificate of attendance.

## Oral presentation

We ask all speakers to approach our technical staff (Roland & Joachim) in the lecture hall during coffee/lunch break before the start of the assigned session to set up the presentation and to check potential adapters.

## Poster presentation

The poster session takes place in the foyer on Sunday (16th June) starting at 18:00. Each presenter will be assigned a poster number and should mount their poster right after registration and remove them until the end of the coffee break on Wednesday.

| Sunday, 16th June 2024 | |
|---|---|
| 12:00 – 14:30 | Registration |
| 14:30 – 14:40 | Opening Remarks |
| 14:45 – 15:45 | Christophe Zimmer (Opening), Würzburg (Germany)<br>"Computational methods for analyzing dynamic biological processes" |
| 15:45 – 16:30 | Coffee Break |
| 16:30 – 18:00 | Flash talks (for Poster session) |
| 18:00 – 21:00 | WELCOME RECEPTION (in-house) & Poster session |

## Monday, 17th June 2024

| | |
|---|---|
| 09:25 – 09:30 | Welcome & Information |
| 09:30 – 10:00 | Christoph Spahn, Würzburg (Germany)<br>"(Re)solving the puzzle: Unraveling bacterial organization using super-resolution microscopy" |
| 10:00 – 10:30 | Izzy Jayasinghe, Sydney (Australia), via ZOOM<br>"Standardising and adapting expansion microscopy for imaging intracellular signalling complexes" |
| 10:30 – 11:00 | Coffee Break |
| 11:00 – 11:30 | Dominic Helmerich, Würzburg (Germany)<br>"Protein-based imaging calibration optical rulers (PicoRuler) for super-resolution microscopy" |
| 11:30 – 12:00 | Ellen Sletten, LA (USA)<br>"Polymethine fluorophores for imaging in the shortwave infrared region" |
| 12:00 – 13:30 | Group picture & lunch |
| 13:30 – 14:00 | Julia Heiby, Jena (Germany)<br>"Advancing proteomics techniques to investigate intricate protein localization patterns" |
| 14:00 – 14:30 | Tamara Girbl, Würzburg (Germany)<br>"New insights into the mechanisms of leukocyte migration through blood vessel walls uncovered by intravital microscopy" |
| 14:30 – 14:50 | Leica Microsystems, Jens Peter Gabriel<br>"Advances in STED Imaging for Gentle Live Cell Nanoscopy" |
| 14:50 – 15:45 | Coffee Break |

| Monday, 16th June 2024 | |
|---|---|
| 15:45 – 16:15 | Ulla Gerling-Driessen, Düsseldorf (Germany)<br>"Multi-functional chemical probes for glycan imaging" |
| 16:15 – 17:15 | Kai Johnsson (Keynote), Heidelberg (Germany)<br>"Recording the physiological history of cells with fluorescent labeling" |
| 18:30 – | Conference Dinner<br>(Wine Tasting in the Residence "Hofkeller") |

## Tuesday, 18th June 2024

| | |
|---|---|
| 09:55 – 10:00 | Welcome & Information |
| 10:00 – 10:30 | Patrick Eiring, Würzburg (Germany)<br>"Deciphering the Surfaceome by dSTORM to improve immunotherapy efficacy" |
| 10:30 – 11:00 | Peter Horvath, Szeged (Hungary)<br>"Solving computational cell biology problems related to light microscopy" |
| 11:00 – 11:30 | Coffee Break |
| 11:30 – 12:00 | Felicitas Schlott, Würzburg (Germany)<br>"Deep-learning based bioimage analysis on a large dataset" |
| 12:00 – 12:30 | Kathrin Doppler, Würzburg (Germany)<br>"Damage to the nodes of Ranvier in peripheral neuropathies" |
| 12:30 – 13:40 | Lunch |
| 13:40 – 14:10 | Johannes Broichhagen, Berlin (Germany)<br>"Chemical Biology Approaches to lighten up Cell Surface Proteins" |
| 14:10 – 14:40 | Philip Tovote, Würzburg (Germany)<br>"Optical methods to study neural circuit function" |
| 14:40 – 15:00 | MMI, Jörg Brühmann<br>"Finding The One: How microdissection and cell picking uncovers the hidden world of single cells" |
| 15:00 – 15:45 | Coffee Break |

| Tuesday, 18th June 2024 | |
|---|---|
| 15:45 – 16:15 | Gil Westmeyer, München (Germany), via ZOOM<br>"Novel probes for non-invasive visualization of molecular and cellular processes in living organisms" |
| 16:15 – 16:45 | Alexander Seitel, DKFZ (Germany)<br>"Development and application of advanced medical imaging techniques" |
| 16:45 – 17:15 | Jonathan Liu, Washington (USA)<br>"Nondestructive 3D pathology and analysis for precision treatments" |
| 17:30 – | Round tables and Discover Würzburg |

## Wednesday, 19th June 2024

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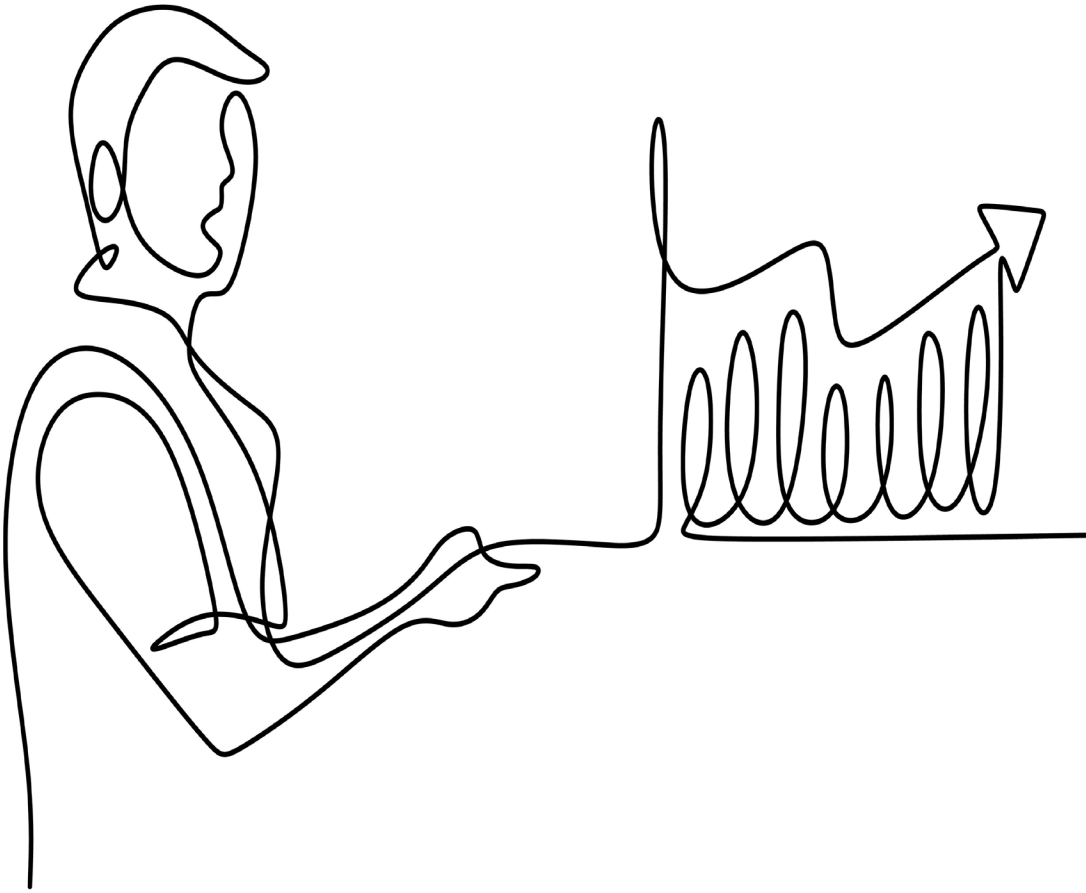
|---|---|
| 09:00 – 09:10 | Information |
| 09:10 – 09:40 | Katrin Streckfuß-Bömeke, Würzburg (Germany)<br>"Use of stem cell derivatives and artificially produced cardiac organoids in the field of cardiovascular precision medicine and drug analysis" |
| 09:40 – 10:10 | Rafael Carazo Salas, Bristol (UK)<br>"Visualising and Predicting Human Stem Cell Differentiation Dynamics" |
| 10:10 – 10:40 | Coffee Break |
| 10:40 – 11:10 | Dagmar Kainmüller, MDZ (Germany)<br>"Developing advanced computational methods for image analysis and machine learning" |
| 11:10 – 11:40 | Sabine Fischer, Würzburg (Germany)<br>"Image-based mechanistic modelling of spatial interactions in multi-cellular systems" |
| 11:40 – 12:10 | Stephan Preibisch, MDZ (Germany)<br>"Analysis and visualization of large-scale, high-dimensional biological imaging data" |
| ~ 13:00 | Closing remarks and Departure |

# Poster Abstracts



# Deciphering the Surfaceome by dSTORM to improve immunotherapy efficacy

[1]Eiring Patrick, [1]Maximilian Steinhardt, [1]Cornelia Vogt, [2]Martin Kortüm, [1]Markus Sauer

[1]Department of Biotechnology and Biophysics, Biocenter, University of Würzburg

[2]Department of Hematology, University Hospital Würzburg

Receptors possess a prominent role as inductors and receivers of signals in all cells. They are essential for any cell pathways including processes like apoptosis and proliferation. Visualization of the "Surfaceome" – the entirety of membrane receptor molecules expressed on the plasma membrane – of a cell remains challenging and quantitative information about addressable molecules and their assembly is largely missing, despite its huge potential impact on targeted medical treatments. Due to its explicit single molecule sensitivity, super-resolution microscopy by dSTORM has the potential to analyze membrane compartments and their assembly with high sensitivity quantitatively, providing detailed insights into present receptor complex formations. Therefore, we compared and optimized different staining protocols and dyes for dSTORM to improve preservation and detection of underlying receptor organizations. This gives us the opportunity to determine stoichiometric changes like multimerizations, which can help to identify activating processes in cells. In terms of medical treatments, this enables the possibility to precisely visualize changes in receptor distribution and accumulation on patient cells and to minimize possible side effects caused by unspecific binding during immunotherapy. Hence, we compared various Multiple Myeloma patients before and after being heavily treated with Daratumumab – an anti-CD38 monoclonal antibody – to get additional insights in CD38 receptor downregulation during therapy. However, in some resistant patients with no CD38 detectable, CD38 persistence could be assessed by dSTORM below the detection rate of flow cytometry, suggesting a Daratumumab efficacy threshold.

# Objective deep-learning based bioimage analysis on a large dataset

Felicitas Schlott, Annemarie Schulte, Beate Hartmannsberger, Alexander Brack, Heike Rittner, Robert Blum
University Hospital Würzburg

Analysis of immunofluorescent images is a widely used technique in the field of translational biosciences. Feature annotation is mostly done manually, however, this holds several challenges. For one, it is time consuming and therefore limits scalability and depth of the sample analyzed. Moreover, it needs to be considered, that bioimage data is in principle ambiguous. Thus, manual image annotation has a certain degree of subjectivity, especially because the experimenter cannot be blinded when the biological effect is obvious.To counteract these disadvantages and improve objectivity and validity of our research, we implemented a deep learning (DL)-based approach for image feature segmentation with the help of the DL tool deepflash2. For that, three experts annotated a training set and we computed a ground truth, to reduce subjectivity. Model ensembles were trained with this computational consensus to predict data on yet unseen bioimages, averting limitations in scalability. Here, we applied this approach to investigate cellular pain resolution phenotypes in rat dorsal root ganglia (DRGs) after nerve injury (Schlott et al., 2024). With eight label-specific model ensembles and advanced thresholding, we analyzed cellular plasticity of sensory neurons, neuron subtypes, satellite glial cells (SGCs), and macrophages in more than 7,500 fluorescence microscope images from large-scale tile- and confocal microscopy. Our data show that peripheral sciatic nerve injury did not cause neuronal loss in the DRG. Changes in cellular plasticity were most prominent in macrophages and SGCs surrounding neurons. Markedly, these effects seen in non-neuronal cells were distinct between female and male animals during ongoing pain resolution. The DL-based models used in this study enabled the analysis of a complex, largescale dataset with multiple cell types to provide new information on sex-specific cellular changes during pain resolution, a novel topic in translational pain research.

# The role of pericytes in cytotoxic T cell transmigration into the infected skin

Cimen Tugce[1], Marilia Fernandes Manchope[1], D. Rowe, Wolfgang Kastenmüller[2], Tamara Girbl[1]

[1]Rudolf Virchow Center for Integrative and Translational Bioimaging, University of Würzburg

[2]Chair of Systems Immunology, University of Würzburg

The efficient recruitment of effector T cell populations, such as CD8+ cytotoxic T lymphocytes (CTLs), to sites of inflammation is essential for host protection. To enter inflamed peripheral sites, circulating CTLs need to breach 2 distinct cellular layers of venular walls i) endothelial cells (ECs) and ii) the surrounding pericyte layer. While the adhesive interactions between CTLs and ECs have been well described, almost nothing is currently known about the mechanisms mediating T cell passage through the pericyte sheath. In order to address this knowledge gap, we studied the transmigration of CTLs into the murine skin following Modified Vaccinia Ankara virus (MVA) infection using a combination of transgenic mouse models, whole-mount confocal and intravital microscopy, and in vitro methods. Our results showed that dermal MVA infection causes extensive morphological modulation of the pericyte layer, which resulted in a significantly decreased pericyte coverage of venular walls, related to enhanced pericyte contractility. Despite the discontinuous nature of the pericyte layer, our data demonstrated that CTLs, after passing the endothelium, engage in extensive physical interactions with pericytes, before fully entering the inflamed tissue. Based on these observations we hypothesized, that pericytes provide an adhesive scaffold for T-cell transmigration into the interstitial tissue. In order to identify the molecular mechanisms mediating the interactions between CTLs and pericytes, we characterized the expression profile of adhesion molecules and chemokines by pericytes. Our analyses showed that pericytes strongly upregulate the expression of the key T cell integrin ligands VCAM-1 and ICAM-1 during MVA infection in vivo. Furthermore, the pro-inflammatory cytokines TNF and IFNγ induced the expression of T cell chemokines CCL2, CCL5, and CXCL16 in primary dermal pericytes. Our current experiments use blocking antibodies and inhibitors to address the function of these pericyte-associated molecules in mediating CTL transmigration into the MVA-infected skin. In summary, our results provide the first evidence of a previously unrecognized cellular interaction between CTLs and pericytes during an adaptive immune response in vivo.

# Optimized genetic code expansion technology for time-dependent induction of adhesion GPCR-ligand engagement

Marcel Streit, Gerti Beliu

Rudolf Virchow Center for Integrative and Translational Bioimaging, University of Würzburg

Fluorescence microscopy plays a crucial part in modern biological research. The constant development of super-resolution techniques steadily improves the capable spatial resolution and enables the visualization of biological structures and dynamics in live cells. Nevertheless, there are still some limitations of those techniques. While the achievable resolution getting higher, a limiting factor of super-resolution microscopy techniques will be the size of the used fluorescent label. The smallest possible label to date is the site-directed introduction of a non-canonical amino acid (ncAA) into the target protein via Genetic Code Expansion (GCE), which includes a strained alkene like trans-cyclooct-2-ene (TCO) and can react with a tetrazine dye in a bioorthogonal click reaction. This reaction is fast and highly specific, allows live-cell labelling under physiological conditions and can be used for the study of protein-protein interactions.

# Towards Spectral Single Molecule Localization Microscopy with a Dual Objective diffraction gratings spectroscope

Mickael Lelek[1], Benoit Lelandais[1], Christophe Zimmer[1,2]

[1]Department of Computational Biology, Imaging and Modeling, Institut Pasteur

[2]Rudolf Virchow Center for Integrative and Translational Bioimaging, University of Würzburg

The development and optimisation of single-molecule localisation microscopy (SMLM) has allowed to image biological structures with nanometric spatial resolution in 3D. However, multi-colour imaging with more than two colors is much more challenging in SMLM than in widefield microscopy. Because fluorophores with non-overlapping spectra have very different photophysical properties and blinking rates, spatial resolution and acquisition time cannot be optimized for multiple colors simultaneously. Fluorophores that share similar photophysical properties, such as the near-infrared dyes AF647, CF660, and CF680, have strongly overlapping spectra and are hence not well separated by conventional emission filters. However, such spectrally close fluorophores can be separated using a dispersive element and a camera (spectroscope). Multicolor SMLM systems have been proposed based on prisms or diffractive gratings. Using gratings, the spatial and spectral information are displayed on the same image, dispensing of the need for an additional optical path, and color can be determined from the distance between the diffraction orders [4]. However, the dispersion of photons into multiple diffraction orders limits the signal to- noise ratio and hence the accuracy of spatial localization and color assignement. To address this limitation, we built a dual-objective SMLM system with two orthogonal spectroscopes in each emission arm. By combining the information from the 2 spectroscopes, it should in theory be possible to improve localization accuracy (by $\sqrt{2}$) and spectral resolution, filter out ambiguous detections and hence improve the resolution and quality of the multicolor reconstructions image. We show results of preliminary validation experiments on composites of single color images and on a multi-labeled sample. With further improvements of image analysis and optics, we believe that this approach will be able to achieve multi-color imaging with spectrally close dyes at high resolution.

# Deciphering peripheral neuropathies in patients by super-resolution microscopy of dermal nerve fibers in skin biopsies

Vinicius da Cruz Neris Geßner[1,2], T. Kremer[1,2], J. Linke[1,2], Hanna .S. Heil[3], L. Appeltshauser[2], Kathrin Doppler[2], Katrin G. Heinze[1]

[1]Rudolf Virchow Center for Integrative and Translational Bioimaging, University of Würzburg

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In peripheral neuropathies (PN), damage to the peripheral nervous system can result from demyelination or axonal impairment. Diagnosing the underlying etiology is often a complex task, with morphological analysis of peripheral nerve fibers being a valuable tool. However, the limited resolution of classic microscopy approaches often hinders accurate diagnosis and a deeper understanding of the molecular mechanisms involved in PN. Furthermore, the invasiveness of nerve biopsies poses challenges for diagnosis and PN research (e.g., difficulty in obtaining healthy controls). Here, we show how to successfully increase resolution and decrease invasiveness by using super-resolution imaging on skin biospsies of PN patients. Our research groups have recently shown that direct Stochastic Optical Reconstruction Microscopy (dSTORM) can identify and quantify the disorganization level in the architecture of nodes of Ranvier proteins and axonal cytoskeleton (Caspr-1, Neurofascin, Beta IV Spectrin, and voltage-dependent channels) in such skin biopsies. This technique has proven effective not only in sural nerve biopsies but also in dermal myelinated nerve fibers obtained through low-invasive skin biopsies from PN patients. Here, we demonstrate the application of dSTORM to obtain detailed images of dermal nerve ultrastructure at the nodes of Ranvier. One challenge encountered was the immunostaining of thicker skin samples, making imaging more susceptible to optical aberrations. We have optimized the imaging pipeline and implemented strategies to image relatively thick skin samples after optimized immunostaining, minimizing optical aberrations for high-contrast images. As a result, we present super-resolved images of the nodes of Ranvier ultrastructure proteins and the axonal cytoskeleton in skin biopsies from patients with PN and ALS compared with healthy human controls. These images showcase the first super-resolved disease-related nerve distortions and correlations in skin biopsies of PN patients, laying the groundwork for using dSTORM as a precise diagnostic tool in the future.

# Enhanced synaptic protein visualization by multicolor super-resolution expansion microscopy

Janna Eilts, Sebastian Reinhard, Nikolas Michetschläger, Christian Werner, Markus Sauer
Department of Biotechnology and Biophysics, Biocenter, University of Würzburg

Understanding the organization of biomolecules into complexes and their dynamics is crucial for comprehending cellular functions and dysfunctions, particularly in neuronal networks connected by synapses. In the last two decades, various powerful super-resolution (SR) microscopy techniques have been developed that produced stunning images of synapses and their molecular organization. However, current SR microscopy methods do not permit multicolor fluorescence imaging with 20 to 30 nm spatial resolution. We developed a method that enables 4-color fluorescence imaging of synaptic proteins in neurons with 20 to 30 nm lateral resolution. We used post-expansion immunolabeling of eightfold expanded hippocampal neurons in combination with Airyscan and structured illumination microscopy (SIM). We demonstrate that post-expansion immunolabeling of approximately eightfold expanded hippocampal neurons enables efficient labeling of synaptic proteins in crowded compartments with minimal linkage error and enables in combination with Airyscan and SIM four-color three-dimensional fluorescence imaging with 20 to 30 nm lateral resolution. Immunolabeling of Synaptobrevin 2 as an efficient marker of the vesicle pool allowed us to identify individual synaptic vesicles colocalized with Rab3-interacting molecule 1 and 2 (RIM1/2), a marker of pre-synaptic fusion sites. Our optimized expansion microscopy approach improves the visualization and location of pre- and post-synaptic proteins and can thus provide invaluable insights into the spatial organization of proteins at synapses.
(Eilts et al., "Enhanced synaptic protein visualization by multicolor super-resolution expansion microscopy," Neurophoton. 10(4) 044412 (25 October 2023). Doi: 10.1117/1.NPh.10.4.044412.)

# Can expansion microscopy provide reliable molecular structure information?

Julia Weingart, Danush Taban, Janna Eilts, Markus Sauer

Department of Biotechnology and Biophysics, Biocenter, University of Würzburg

The field of microscopy enables the world to see beyond the limits of human vision. Expansion microscopy (ExM) has emerged there as a transformative technique, offering a unique approach to further overcome traditional resolution limits. By physically expanding biological samples, ExM holds promise for providing enhanced spatial resolution in imaging studies. The combination of super-resolution microscopy with expansion protocols, enables to an even higher resolution of cellular structures. Nevertheless, when zooming in even deeper than previously possible through super-resolution microscopy alone, it is of great importance to look at the results thoroughly. The limitations and challenges associated with ExM and super-resolution microscopy must be considered to assess the reliability of ExM in preserving and accurately visualizing the native molecular architecture. Therefore, both isolated protein structures like IgM (around 40 nm) and cellular compounds such as clathrin-coated pits (60-200nm), whose shapes are already known from electron microscopy, were used to investigate this question.

# Visualization of Cas9 proteins in interaction with RNA and DNA applying super-resolution microscopy methods

Linda Stelz, Markus Sauer

Department of Biotechnology and Biophysics, Biocenter, University of Würzburg

Since its discovery, the CRISPR-Cas system has been widely embraced by the gene editing community as a powerful tool to understand and ultimately treat certain types of cancer and genetic diseases. The CRISPR-associated Cas protein can be programmed by a single-guide RNA to bind to specific sequences of the DNA. Depending on the properties of the Cas molecule it can either cause double or single strand breaks, as well as just attach to the DNA without any further enzymatic processes taking place. For a better understanding of the mechanisms of the Cas protein interactions, microscopy methods such as AFM (Atomic force microscopy) or Electron microscopy (EM) have been mainly used. Here we use multicolor super-resolution fluorescence microscopy in combination with Expansion Microscopy (ExM) to visualize the molecular state of the Cas9 molecule bound to the sgRNA and the DNA target at virtually molecular resolution.

# Let's get photophysical! Simulating Cy5 photophysics during sub-10 nm dSTORM experiments

Vincent Ebert, Markus Sauer, Sören Doose

Department of Biotechnology and Biophysics, Biocenter, University of Würzburg

Direct Stochastic Optical Reconstruction Microscopy (dSTORM) experiments with cyanine 5 (Cy5) in defined positions have shown shortened nonfluorescent OFF states the smaller the distances. We developed a simulation machinery that is capable of accurately reproducing virtually any photophysical process including energy transfers within systems of a small number of fluorophores. Our group is seeking to construct an extended photophysical model of Cy5 in the context of dSTORM that explains experimentally observed data. The comprehensive model predicts the distance dependent blinking pattern, providing the basis for drawing conclusions about interfluorophore distances below 10 nm in experimental settings.

# Molecular resolution fluorescence imaging by Ex-SMLM

Danush Taban[1], Dominic Helmerich[1], Made Budiarta[2], Luke Lavis[3], Gerti Beliu[2], Ulla Gerling-Driessen[4], Silvio Rizzoli[5], Felipe Opazo[5], Markus Sauer[1]

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[3]HHMI/Janelia Farm

[4]Institute of Macromolecular Chemistry, University of Freiburg

[5]Institute of Neuro- and Sensory Physiology, University of Göttingen

Current advances in super-resolution microscopy enable the detection of single molecules with nanometer localization precision. However, translating such high localization precisions into sub-10 nm spatial resolution in biological samples remains a demanding challenge. Here, we present the next crucial step towards achieving true molecular resolution fluorescence imaging by combining Expansion Microscopy (ExM) with single-molecule localization microscopy (Ex-SMLM). Our new simplified and fast expansion protocol can be tailored to a wide range of samples, ranging from nuclear pore complexes (NPCs) via tubulin filaments to proliferating cell nuclear antigen (PCNA) ensuring ultrastructure preservation of the native structures. The combination of ExM and new spontaneously blinking dyes circumvents the need for special blinking buffers or complex re-embedding protocols thus enabling SMLM in conventional hydrogels in the absence of special switching buffers.

# Visualization and quantification of the interaction of CAR-T-cells with tumor cells

Nicole Seifert[1], Leon Gehrke[2], Rick Seifert[1], Sören Doose[1], Thomas Nerreter[2], Michael Hudecek[2], Markus Sauer[1]

[1]Department of Biotechnology and Biophysics, Biocenter, University of Würzburg

[2]Lehrstuhl für Zelluläre Immuntherapie, Medizinische Klinik und Poliklinik II, University Hospital Würzburg

In Chimeric Antigen Receptor (CAR)-T-cell therapy, T-cells are modified with a CAR that targets membrane receptors on tumor cells such as CD19, CD20 and BCMA. To visualize the interaction between CAR-T-cells and tumor cells it is of utmost important to ensure efficient labelling of the CAR. Therefore, different labelling methods such as dye conjugated antibody, idiotype antibody and antigen were compared in co-culture by confocal fluorescence microscopy. For receptor quantification all labelling methods were also imaged at a higher spatial resolution using direct stochastic optical reconstruction microscopy (dSTORM). With this single molecule sensitive method even low abundant receptors can be detected and quantified, and a more detailed cluster analysis performed. In addition, two color dSTORM measurements can be used to visualize the colocalization and distribution of two receptors. With these methods it is possible to visualize CAR-T-cells and improve CAR-T-cell therapy. For example, it is possible to visualize the amount of CARs organized in nanodomains dependent on different transfection methods or different CAR constructs.

# High throughput quantification of membrane receptors for immunotherapy

Rick Seifert, Nicole Seifert, Sören Doose, Markus Sauer

Department of Biotechnology and Biophysics, Biocenter, University of Würzburg

Expression level of membrane receptors is of great importance for immunotherapy of myeloma patients. The expression levels of the target receptors are normally determined by Flow Cytometry (FC). Low expressed target receptors are important for immunotherapy, which often cannot be detected with FC due to its limited sensitivity. Using direct Stochastic Optical Reconstruction Microscopy (dSTORM), a Single Molecule Localisation Microscopy (SMLM) technique, even low expressed target receptors can be quantified. However, only a small number of cancer cells can be analysed, as many steps are currently done manually. [1] We have developed an automated dSTORM workflow to quantify many patient cells with high sensitivity. To quantify different membrane receptors and detect colocalization, we have extended this system with multicolor dSTORM imaging. The number of cells analysed can be further increased by single molecule sensitive methods such as Re-Scan confocal microscopy (RCM) or total internal reflection (TIRF) microscopy [2]. However, these techniques cannot achieve as high resolution as dSTORM. In addition to the dSTORM based workflow, we are working on an RCM- and TIRF-based workflow for high throughput receptor quantification. All three techniques are combined in a single microscope, so that many cells can be screened with RCM / TIRF, and selected cells can be further analysed with a high spatial resolution in dSTORM mode. With this setup patient samples from myeloma patients can be analysed in the future with good statistics and high sensitivity.

[1] Nerreter T, Letschert S, Götz R, et al. Super-resolution microscopy reveals ultra-low CD19 expression on myeloma cells that triggers elimination by CD19 CAR-T. Nat Commun. 2019;10:3137.

[2] De Luca GMR, Breedijk RMP, Brandt RAJ, et al. Re-scan confocal microscopy: scanning twice for better resolution. Biomed Opt Express. 2013;4:2644.

# Chemical probes for selective lysosomal imaging

Simon Walber, Lukas Zeipelt, Ulla Gerling-Drießen
Institute of Macromolecular Chemistry, University of Freiburg

Chemical probes for selective lysosomal imaging Lysosomes, as the central cellular hub for macromolecule degradation and recycling, are involved in the degradation of endogenous and foreign (pathogenic) substrates. They also regulate proper cellular function by providing resources for other organelles. Defects that lead to an impairment of lysosomal function are summarized as Lysosomal storage diseases (LSDs). The dysfunction of lysosomes can lead to the accumulation of incompletely digested substrates within the lysosome, impaired communication with other organelles, cell damage and even result in cell death. Currently more than 70 different types of LSDs have been identified. Although, individually the occur rarely, together these defects affect 1 in 5,000 live births. Even though gene and enzyme replacements therapies have been successfully established for some LDS, research is still underrepresented for most of these rare disorders. Gaining deeper insight into cellular consequences of individual LSDs, would require a detailed characterization of alterations of the particular lysosomal components. However, the selective organelle-specific characterization of proteins and other macromolecules is still very challenging with the currently available technology. However, this understanding would benefit not only a better understanding of LSDs but also other, more common diseases, such as cancer and neurodegenerative diseases that are associated with lysosomal function. Here, we present the development of novel chemical probes that can selectively target molecules within lysosomes and enable their visualization, isolation and characterization. Active transport into the lysosome is achieved via the cation-independent mannose-6-phosphate (Man6P) receptor. We have developed a new and efficient synthesis method for the selective functionalization of saccharides, which can be used to efficiently produce Man6P-oligomers and polymers. The Man6P ligand, which ensures selective lysosomal uptake, is further equipped a specific azidocoumarin building block that gains in-situ fluorescence upon photo-induced crosslinking to target proteins in close proximity. The probe also contains a biotin unit that serves as affinity tag for isolation and subsequent characterization of the crosslinked lysosomal targets. We will use these lysosomal-specific probes to study lysosomal abnormalities in the context of LSD and associated diseases.

# Lightening up the Synapse: Sylites

Christiane Huhn, Sheng-Yang Ho, Clemens Schulte, Vladimir Khayenko, Katherina Hemmen, Thomas-Otavio Peulen, Sebastian Bothe, Ivan Talucci, Anna-Lena Wießler, Lars Schönemann, Christian Werner, Carmen Villmann, Martin Hruska, Hermann Schindelin, Kristian Strømgaard, Katrin Heinze, Johannes W. Hell, Hans M. Maric

Rudolf Virchow Center for Integrative and Translational Bioimaging, University of Würzburg

The spatiotemporal organization of the postsynaptic density is a fundamental determinant of synaptic transmission, information processing and storage by the brain. Current limitations in the analysis of excitatory and inhibitory synapses at nanometer-scale resolution arise from the requirement of introducing fluorescent tags through genetic engineering or the multistep application of large antibodies or nanobodies. In addition, currently available affinity probes and intracellular delivery strategies impose major challenges on functional studies either lacking the necessary penetration into the tissue, incomplete or excess cytoplasmic delivery or limited contrast. Targeting the major hallmark proteins of the neuronal inhibitory and excitatory synapses, Sylites1 were developed as compact fluorescent probes with low nanomolar affinity and proteome-wide selectivity. The chemical probe design minimizes dye interference and enables simplified visualization of synapses with optimized contrast and within deep tissue due to their compact design. The combination with novel cell penetrating tools enables perturbance-free functional analysis of genetically unmodified living neurons.

# 3D-Live-cell imaging and biophysical analysis of the highly dynamic endosomal system in *Trypanosoma brucei*

Thomas Müller, Markus Engstler

Chair of Cell and Developmental Biology, Biocenter, University of Würzburg

As an extracellular parasite, Trypanosoma brucei is highly dependent on its diffusive surface coat to shield itself from the immune system of the host organism. The high membrane flux is governed by constant endo- and exocytosis at the flagellar pocket. Recent findings from our group employing electron and super-resolution microscopy techniques, have unveiled the three-dimensional architecture of the endosomal system. This system comprises a cohesive membrane network with distinct subdomains that exhibit enrichment of classical markers associated with early, late, and recycling endosomes1. As the study was conducted using fixed cells, dynamic measurements were not feasible. Consequently, we expanded our single-molecule microscope to incorporate 3D capabilities using astigmatism for imaging various markers associated with the endosomal pathway. However, integrating this additional dimension necessitated the development of a new analysis pipeline. While tools for localizing2 and linking tracks3 in both two and three dimensions exist, visualization and analysis software typically focus on two-dimensional data4. To simplify the exploration and analysis of three-dimensional track data, we developed ThirdPeak— a MATLAB-based software with a user-friendly graphical interface5. During preprocessing, users can apply quality filters, correct for drift, and automate the tracking process. Once the results are validated, in-depth analysis becomes possible. By employing this software, we were able to unveil the dynamics within the endosomal system of Trypanosoma brucei. Our observations indicate that markers for early and late endosomes exhibit relatively confined localizations, punctuated by intermittent rapid translocations. In contrast, the marker for the recycling endosome displays slower and more constrained movement, hinting at an association with molecular motor-driven transport. Our next objective is to delve deeper into the analysis of the diffusional data, with a particular emphasis on anomalous diffusion, which is prevalent in confined yet dynamic cellular compartments. To achieve this, we intend to leverage neural networks to classify the various diffusional behaviours and elucidate their underlying biological significance6. To our knowledge, this study represents one of the most comprehensive investigations into the dynamics of the endosomal system in a eukaryotic organism to date.

# Integrating spectroscopy and imaging data: Bridging molecular information to microscopic scales.

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Jointly utilizing molecular information and spectroscopic data on microscopic scales is an ongoing challenge in molecular microscopy. We aim for a joint description of multiparameter fluorescence image spectroscopy (MFIS) and single molecule multiparameter fluorescence detection (MFD) spectroscopy data using molecular system representations. To jointly describe diverse spectroscopic and imaging data, we developed software (tttrlib and ChiSurf) for representing our data in probabilistic graphical models. Our software can utilize additional structural data through the integrative modeling platform (IMP). By Bayesian modeling using additional structural biology information, we determine affinity constants of molecular assemblies and conformations of proteins in living bacterial systems. Combining spectroscopic in vitro, image spectroscopy, and structural molecular data paves the way towards in situ integrative molecular biology.

# High-content 3D fluorescent imaging for fate mapping of the clonal immune response in the context of myocardial infarction

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Brainbow was originally developed by a team led by Jeff W. Lichtman in 2007 to distinguish neurons in mouse from neighboring neurons using fluorescent proteins. Today, this concept of randomly expressing different ratios of red, green, and blue fluorescent proteins in individual cells for color mapping is widely used even beyond neuroimaging. Here, we use a cassette of the four fluorescent proteins M-mCerulean, hrGFP II-NLS, EYFP, and tdimer2 to image B cell-based clonal immune responses in the heart and lymph nodes after myocardial infarction with different color combinations. The two main bottlenecks are to find a suitable spectral unmixing to disentangle the overlapping fluorescence emission spectra, and a gentle optical tissue clearing protocol that makes the opaque tissue glassy without compromising the brightness of the fluorescent proteins. Since optical clearing may never be perfect under these circumstances, we chose a combination of light-sheet fluorescence microscopy (LSFM) of the whole organs and confocal as well as two-photon microscopy of tissue sections for high-resolution large-content imaging. Here, we present the imaging pipeline with optimized tissue clearing, multi-color LSFM and advanced image analysis using PICASSO[1] unmixing. To reduce animal experiments and refine our pipeline we established a 3-dimensional spheroid calibration assay involving the fluorescent proteins of interest. With all main challenges mastered, we can now image B cell-based clonal immune responses in the heart and lymph nodes in the context of myocardial infarction. Our studies in Nur77GFP-lymph nodes and hearts provide first insight into the localization and number of activated B cells after MI. Next, tissue sections give us an idea about B cell spatial distributions and densities in brainbow lymph nodes. Whole lymph node and heart 3D-imaging in brainbow mice will eventually provide the full picture of the immune response after myocardial infarction.

# Towards understanding the role of transcription factor oligomerization in regulating gene expression in live cells

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Transcription Factors (TF) are often multidomain proteins that gate access to specific DNA sequences crucial for gene activation or repression. The P-family of the Forkhead-box TFs (FoxP) contains a highly conserved Forkhead-box DNA binding (FKH), a leucine Zipper (ZIP) domain connected by an unstructured linker, and a low complexity domain towards the N-terminal. The FKH and ZIP domains are known to dimerize, but the exact mechanism, dynamics, and domain organization, under physiological conditions remain evasive. Our aim is to study the assembly of FoxP1 dimers and potential oligomers in live cells under physiological conditions. Here, we would like to delineate the contribution of the individual structured domains, FKH and ZIP, and the unstructured N-terminal, poly-Q rich region to the dimerization and thus, gene expression regulation. We study the assembly of FoxP1 dimers by multiparameter fluorescence imaging spectroscopy. Experiments on full-length and truncated wild-type and point-mutated FoxP1 domain variants (ZIP-FKH, FKH and R514H, A500P, respectively) tagged by eGFP or mCherry in live cells. An automated analysis pipeline using e.g. tttrlib processes fluorescence-polarization resolved confocal PIE-FRET-FLIM data to determine mean fluorescence lifetime and fluorescence anisotropies and integrates machine learning based segmentation, and nuclei classification to spatial resolved compositions of molecular complexes. Our preliminary results show that the Q-rich domain seems to play an important role in FoxP dimerization and oligomerization while the R514H mutation, which abolishes DNA binding, seems to lead to nuclear condensation disrupting the nuclear organization.

# Subcellular distribution of IP3 and ryanodine receptors (IP3R/RyR) in hippocampal neurons revealed by 8-fold Expansion Microscopy

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Ca2+ release from the endoplasmic reticulum (ER) plays a crucial role in the amplification and propagation of intracellular Ca2+ signals, which activate cytoplasmic and nuclear Ca2+-dependent signaling pathways required for synaptic plasticity. This Ca2+ release from the ER is possible through the channels IP3 and ryanodine receptors (IP3Rs/RyRs), and three isoforms (1-3) of each channel are present in mammals, with IP3R1, RyR2, and RyR3 being the most functionally relevant and expressed isoforms in hippocampal neurons. It has been shown that RyR3 is enriched in dendritic spines of these neurons, while RyR2 is absent in these structures,and IP3R1's presence is debated. Additionally, ryanodine- and IP3- sensitive Ca2+ stores have been probed to be functionally distinguishable from each other through their refilling mechanisms. Here, we aim to give sense to this apparent specialization of IP3R1/RyR2/RyR3 by studying the subcellular distribution of these channels using 8-fold Expansion Microscopy and Airyscan fluorescence imaging. We immunostained mice hippocampal cultures for these proteins, along with CaV2.1, PSD95, and Bassoon, and we als used the membrane probe mCLING. We were able to resolve individual ER cisterns in the soma of neurons (FWHM of 60-80 nm of diameter), which we further analyzed for IP3R1/RyR2/RyR3 distribution. Furthermore, when we focused on dendrites, dendritic spines, and synapses, we found that IP3R1 is present in dendritic spines, and enriched in the vicinities of the scaffold protein PSD95, while RyR3's presence is scattered in dendritic spines. Future research will help to explain the functional advantages of this specialization and localization, and how any disturbance of this complex and fined-tuned network of intracellular Ca2+ stores lead to diseases.

# Re-Engineered Pseudoviruses for Precise and Robust 3D Mapping of Viral Infection

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Engineered vesicular stomatitis virus (VSV) pseudotyping offers an essential method for exploring virus–cell interactions, particularly for viruses that require high biosafety levels. Although this approach has been employed effectively, the current methodologies for virus visualization and labeling can interfere with infectivity and lead to misinterpretation of results. To address this limitation, we introduced an innovative approach combining genetic code expansion (GCE) and click chemistry with pseudotyped VSV to produce highly fluorescent and infectious pseudoviruses (clickVSVs). These clickVSVs enable robust and precise virus–cell interaction studies without compromising the biological function of the viral surface proteins. We evaluated this approach by generating VSVs bearing a unique chemical handle for click labeling and assessing the infectivity in relevant cell lines. Our results demonstrate that clickVSVs maintain their infectivity post-labeling and present an efficiency about two times higher in detecting surface proteins compared to classical immunolabeling. The utilization of clickVSVs further allowed us to visualize and track 3D virus binding and infection in living cells, offering enhanced observation of virus–host interactions. Thus, clickVSVs provide an efficient alternative for virus-associated research under the standard biosafety levels.

# The vascular tree of the femoral bone marrow

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The bone marrow (BM) is richly vascularized tissue, and the main physiological site for adult hematopoiesis. It has been recently shown that cardiovascular diseases can alter the bone marrow vasculature. Here, we generate a 3D reconstruction of the whole bone-marrow vasculature of a murine femur from wild type mice after blood loss, before blood loss and old mice from C57BL/6J mice. First, for fluorescence imaging we have stained the vascular system of fifteen femurs using anti-CD31 and anti-CD105 antibodies labeled with Alexa647 and Alexa546, respectively. Using low magnification light sheet fluorescence microscopy (LSFM), we have acquired tile scans of whole bones, and identified the CD105 positive vessels, also termed as the small vessel vasculature. Here, we only analyzed 4 tile sections corresponding to the diaphysis of the mouse femur. They were analyzed through a combination of image-segmentation and machine learning toolkits. The graph structure of the skeletonized small vessel vasculature was parameterized to identify the degree of conservation of the architecture across tiles in a single bone and hallmark differences between bones of differing physiological subsets. These parameters are number of branching points, branch length and tortuosity. Preliminary analysis of these parameters revealed that in healthy mice the small vessel vasculature is indeed largely conserved across bones of the same physiological background and across slices of the same bone. Some hints of characteristic differences regarding network properties of the microvasculature across different physiological subsets have materialized from preliminary analysis of the data.

# Visualizing trans-synaptic nanocolumns by Expansion Microscopy

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High fidelity neurotransmission in the millisecond range is facilitated by ultrastructural arrangement of pre- and postsynaptic proteins. Recent reports elucidated existence of trans-synaptic nanocolumns and their importance in neuropathology. For instance, in a mouse model for epilepsy, trans-synaptic alignment of PSD95 with LGI1 (leucin-rich glioma inactivated protein 1), a neuronal secreted protein, was significantly impaired. Visualization of trans-synaptic nanocolumns so far relied on super-resolution microscopy approaches like 3D localization microscopy. In this work we reasoned that sample enlargement via a single 8x sample expansion step (Expansion Microscopy) in combination with a straightforward super-resolution microscopy approach (Airyscan imaging) without reconstruction artifacts helps in visualization of trans-synaptic nanocolumns. Using the combination of these super-resolution microscopy approaches we visualize paramount trans-synaptic nanocolumn pairs along with additional labeling of ultrastructural context via whole-proteome staining applying NHS-ester dye conjugates. Furthermore, we show LGI1 protein localization to the synaptic cleft juxtaposed to trans-synaptic nanocolumns. This combination of super-resolution imaging approaches and freely available code for our analysis algorithm will facilitate elucidation of trans-synaptic organization and their integrity in health and disease e.g. in limbic encephalitis with autoantibodies targeting LGI1.

# PicoRuler: Protein-based ruler for sub-10 nm fluorescence imaging

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Super-resolution microscopy (SRM) greatly enhanced the spatial resolution of fluorescence microscopy. Today, novel SRM methods achieve spatial resolutions below 10 nm. To showcase the resolving power of these methods, reliable and biocompatible reference structures are required. Currently, the demonstration of sub-10 nm spatial resolution remains dependent on DNA origami. However, its suitability as a reference system is debatable. Therefore, here we introduce PicoRuler (Protein-based Imaging Calibration Optical Ruler), multilabel oligomeric proteins designed as advanced reference samples for sub-10 nm fluorescence imaging. First, PicoRuler was created based on the homotrimeric protein Proliferating Cell Nuclear Antigen (PCNA). PCNA was site-specifically labeled through Genetic Code Expansion (GCE) (Norb-K) and click chemistry (H-Tet-Cy5) to produce a protein nanoruler with 6 nm interfluorophore distance. The performance of PCNA PicoRuler was then demonstrated through photoswitching fingerprint analysis and DNA-based Point Accumulation for Imaging in Nanoscale Topography (DNA-PAINT). Photoswitching fingerprint analysis distinguished singly- and triply-labeled PCNA PicoRuler and DNA-PAINT resolved the three fluorophores at 6 nm distance. To achieve greater labeling flexibility, we designed a new PicoRuler based on the Circular Tandem Repeat Proteins (cTRPs). The resulting cTRP PicoRulers enabled the incorporation of a higher number of fluorophores at different distances within 10 nm radius. Comprehensive characterizations of the new cTRP PicoRulers through photoswitching fingerprint analysis and DNA-PAINT are currently underway.

# A distinct phase of CD8 T cell priming discovered with intravital microscopy that selects high affinity clones and scales effector cell formation

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T cell priming in lymph nodes is characterized by three distinct phases 1. A search phase with short-term interaction with Dendritic cells (DC), the activation phase with long-term interactions and finally disengagement from DC 2. During this third phase T cells recommence their migratory activity, proliferate and are believed to differentiate autonomously 3,4. Using intravital microscopy, we showed here that unexpectedly a fraction of activated CD8 T cells re-engaged with DC and formed stable clusters in specific microdomains for hours, which we term phase 4. Phase 4 enabled optimal access to paracrine IL-2 signals, which was required for clonal expansion and effector cell differentiation. Antigen-specific CD4 T cells provided IL-2 in a stop-and-go migration pattern, while Treg cells locally consumed IL-2 without cellular arrest. Importantly, low affinity CD8 T cell clones failed to arrest in these microdomains and hence to access IL-2 signals. Thus phase 4 orchestrates the cellular dynamics of the producer, receiver and regulator of key paracrine signals in spatially and temporally distinct niches. Thereby, it facilitates affinity selection of the most potent CD8 T cell clones, reminiscent of the germinal center reaction. Our results revise the current understanding of CD8 T cell differentiation and have implications for vaccinations and cellular immunotherapies.

# Live imaging and cell tracking: applications for 3D and 2.5D fiber-hydrogel composites

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The field of biofabrication has been rapidly growing over the last decade. As improvements in 3D printing and culture technologies lead to increasingly complex fabricated tissue models, there is high demand for the development of proper methods for characterization. Imaging and microscopy techniques are of particular importance for the field since they allow researchers to interrogate 3D samples at cellular and subcellular levels. We have developed tools that focus on the use of live imaging both for long and short periods of time and analyze the cell migration and behavior. In our latest study, motor neuronal-like cells were co-cultivated with microfibers that differ in shape, size and composition. We studied how these cells interact with different kind of mechanical and topographical cues, presented by various microfibers. Time-lapse videos were recorded over a period of 3 days with an incubator live-imaging microscope (ZenCell Owl). It was possible to identify different interactions elicited from the cells towards the fibers. Semi-manual tracking was used to track cell-fiber movement among restricted frames of interest. That allowed quantifying and determining the diverse interactions with distinct fiber types to describe interactions with various fibers; but for example, also to study tumor cell migration and contact within its microenvironment. Confocal live imaging has been performed in fiber-reinforced 3D hydrogel composite tri-cultures of glioma cells with cortical neurons and astrocytes. The aim is to utilize similar approaches to study how glioma cells would migrate within the hydrogel. These analyses will allow us to understand if tumor cells utilize surrounding cells or fibers as guiding cues for migration. Altogether, utilizing live imaging techniques and cell tracking systems has proved to be an essential tool to study important aspects in the development of complex 3D in vitro models.

# Fast volumetric single-molecule localization microscopy of endogenous cell membrane receptors

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DNA-based points accumulation for imaging in nanoscale topography (DNA-PAINT) excels among various existing single-molecule localization microscopy (SMLM) modalities with the possibility to achieve sub-10 nm spatial resolution using a conventional widefield/TIRF microscope (1), easy multiplexing via sequential imaging of targets of interest with different imager strands (exchange-PAINT) (2), and resilience to photobleaching of fluorophores. However, in DNA-PAINT, background fluorescence emerging from unbound imager probes limits the probe concentration to <0.5 nM, thereby reducing the overall imaging speed. To this end, we introduce a novel class of dual-labeled self-quenching imager variant exhibiting strong fluorescence quenching when unbound, enabling imaging at higher probe concentrations with a ~15-fold increase in imaging speed (3). For the proof-of-concept, we demonstrate this new approach, two-dye imager (TDI)-DNA-PAINT for 2D imaging of cellular microtubule networks and DNA origami nanostructures. Furthermore, we combine TDI-DNA-PAINT with lattice light-sheet microscopy (LLSM) (4) for fast volumetric imaging of cellular microtubule networks. Finally, using LLSM-TDI-DNA-PAINT, we visualize the whole-cell nanoscale distribution of endogenous CD20 receptors in B cells and the rearrangement of CD20 into filamentous structures after treatment with the therapeutic monoclonal antibody Rituximab.

# Path3D: A comprehensive pipeline for non-destructive 3D pathology

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Conventional slide-based histology has remained the gold-standard for microscopic evaluation of clinical and preclinical tissue specimens for the past century. In a typical clinical workflow, fixed tissues are cut into thin sections on glass slides, stained, and manually viewed by a pathologist. Despite being a mainstay of anatomic pathology, slide-based histology workflows face several limitations that impact critical clinical decision making. First, pathologists typically examine only a few thin (4 - 5 μm) sections from a tissue specimen due to the time and manual effort required to prepare and review each slide, representing < 1% of the total tissue volume. Two-dimensional sections also fail to capture the 3D structure of many tissues, such as complex vascular or glandular networks that are helpful for grading diseases like prostate cancer. In addition, the physical sectioning required for slide-based histology is inherently destructive to tissue, complicating the downstream molecular analyses that are increasingly used in clinical practice. Non-destructive 3D pathology is an alternative approach proposed by our lab and others in which tissue specimens are optically cleared with gentle reversible protocols to render them transparent to light, fluorescently labeled, and imaged with volumetric microscopy. 3D pathology addresses many of the shortcomings of slide-based histology: the entire tissue volume can be imaged and analyzed, 3D tissue structure can be interrogated, and intact specimens are preserved for archival purposes or downstream analysis. A growing number of proof-of-concept studies suggest that 3D pathology could provide clinically relevant information. Examples include resolving ambiguities in prostate cancer grading [1-3] and more accurately characterizing lymph node metastasis size [4], both of which have implications for treatment planning. While most work to date has used relatively small sample sizes, clinical adoption of 3D pathology will require large-scale studies. A major requirement for large studies is producing consistent, high-quality data across 100s to 1000s of volumetric specimens, which is nontrivial and requires careful tuning of a variety of parameters. To accelerate the adoption of 3D pathology for researchers and clinical labs, we present Path3D, an end-to-end pipeline for non-destructive 3D pathology [5]. We describe the tissue preparation, imaging, and data processing steps needed to go from formalin-fixed paraffin embedded (FFPE) tissues to 3D datasets ready for human review or computational analysis. We also cover quality control procedures that are essential for optimizing and maintaining image quality in large sample cohorts.

# Towards Robust Oligomer Characterization by Fluorescence Correlation Spectroscopy

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In Fluorescence Correlation Spectroscopy (FCS), analysis of fluorescence fluctuations from molecules in a small observation volume reveals mobility and relative concentrations of molecules. In theory, FCS should be a powerful technique for studying self-assembly, a key principle in structure and function of living systems (e.g., cytoskeleton filaments). In practice however, it often performs weakly in characterizing mixtures of self-assembling oligomers. There are multiple reasons for that, which are inherent to the statistical properties of the autocorrelation function usually analysed in FCS. Reviewing existing literature and adding our own investigations, we offer insights on where this weakness comes from as well as suggestions to improve on it in both experiment and data analysis. We present benchmarking results from our efforts to combine previously suggested and new ideas into a common analysis framework. Our results can help make FCS a more robust technique for characterizing self-assembling systems and further democratize the technique.

# Axonal modulation of sensory neurons upon NFG stimulation

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Chronic pain is a debilitating condition lowering the quality of life of patients. Treatment options are quite limited, mostly focusing on symptomatic treatment. Pain is signalled via nociceptive neurons located in the DRGs from the periphery to the spinal cord This causes a change in RNA messaging and protein expression. While transcriptomic and proteomic alterations are known in the cell bodies of DRGs, the axonal changes are still poorly understood, focusing on a small number of transcripts. In this project we have cultivated sensory neurons in microfluidic chambers. This has allowed us to extract pure axonal material after stimulation with NGF, a neurotrophic factor that can inducing inflammatory pain. In our data we have seen an dysregulation of signalling for the immune response.

# Dynamics of BDNF-TrkB signaling

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' Neurotrophic factors are group of important regulatory biomolecules which affect neuronal survival, outgrowth, synapse formation, and many other cellular functions. Nerve Growth Factor (NGF) stimulation causes a sensitization by local regulation of channels and receptors at the plasma membrane. Further, the NGF-TrkA-complex also works as a transcription factor, regulating different genes (including BDNF) to dynamically regulate neuronal activity. Since the neurotrophic factors exhibit local activity, and can therefore have differential effects within neurons. Dorsal Root Ganglia (DRGs) have been previously characterized using broader contexts, especially focusing on the soma. Corresponding changes in the axons and nerve endings, however, are still poorly understood. Such local changes due to the activity of NGF are implicated in the chronification of pain. Here we attempt to build a framework to elucidate these local changes by using a constellation of physical and biochemical cell culture techniques, and imaging modalities.

# Platelets drive thrombo-inflammation through Platelet-derived Integrin and Tetraspanin-enriched Tethers (PITTs)

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Background: Over the past decade, it has become increasingly evident that platelets serve essential roles beyond hemostasis. Our laboratory has recently discovered that resting platelets can rapidly reorganize the entire pool of their main adhesion receptor, integrin αIIbβ3, along with its associated tetraspanin (CD9) and a signaling machinery into distinct membrane mircodomains, which serve as building blocks for a new organelle, that we termed Platelet-derived Integrin and Tetraspanin-enriched Tethers (PITTs). PITTs display a unique composition of membrane proteins, contains its own organelles, and PITTs promote thrombo-inflammation by interacting with neutrophils and endothelial cells. To study the morphology and molecular composition of this new organelle, we utilized a wide range of modern microscopy techniques. Aims: Decipher the molecular machinery that mediates αIIbβ3 regulation and analyze the characteristics of PITTs with different microscopy methods to get detailed insights into their structure and function. Methods: direct Stochastic Optical Reconstruction Microscopy (dSTORM) - Single-Molecule Localization Microscopy. Flow chamber microscopy. Scanning Electron Microscopy (SEM). Confocal Microscopy. Results: Our laboratory has established an in vitro system allowing the formation and visualization of PITTs by using micropatterning-based flow adhesion assay. The forming PITTs were analyesed by different microscopy techniques including CLSM, dSTORM super-resolution microscopy and SEM. We demonstrated virtually unrestricted lateral mobility of αIIbβ3 receptors in the plasma membrane of resting platelets, which was also confirmed by in vivo experiments in mice expressing GFP-tagged αIIb. We identified a so-far undescribed structure at the platelet proximal region of forming PITTs that appear to function as a sorting machinery to target membrane proteins and cargo into these structures while excluding other molecules and subcellular structures such as mitochondria or alpha-/dense granules. With the application of confocal and super-resolution dSTORM analysis, we analysed principal steps and molecular mechanisms underlying PITTs formation. Our findings suggest that PITTs are likely formed without initial cytoskeletal involvement, although actin filaments appear to polymerize into formed PITTs structures. By taking the advantages of each of these techniques, we were able to decipher first constituents of PITTs. Conclusion: This study reveals a previously unrecognized mechanism involving the reorganization of αIIbβ3 and flow dependent (probably actin-independent) formation of αIIbβ/CD9 enriched tethers structures (PITTs) that constitute novel mechanisms underlying platelet-driven thrombo-inflammation.