NENCKI Microscopy Workshop, 28th – 31st May 2014, Nencki Institute of Experimental Biology, Warsaw, Poland

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Wednesday, May 28th

16.00 – 19.00 **Registration, snacks and refreshments**

Thursday, May 29th

8.55 – 9.00 Opening of the Workshop (G.M. Wilczynski, J. Wlodarczyk, T. Bernas)

9.00 – 9.45 **Ireneusz Grulkowski** "Multidimensional interferometric imaging and metrology in biomedicine – from microscopy to mesoscopy"

9.45 – 10.30 **Yannick Schwab** "Correlative Light and Electron Microscopy: taking snapshots of the living at the ultrastructural level"

10.30 – 10.50 **Matthia Winter-Karreman** "Lights Will Guide You: Tracking Invasive Tumor Cells with Correlative Intravital Fluorescence Microscopy and Electron Microscopy"

10.50 – 11.10 Coffee break

11.10 – 11.55 **Evgeni Ponimaskin** "Regulation of receptor functions by heterodimerization of serotonin receptors"

11.55 – 12.40 **Jonas Ries** "Superresolution Microscopy of Protein Structures in Situ"

12.40 – 13.00 **Adriana Magalska** "Loss of neuronal 3D chromatin organization causes transcriptional and behavioral deficits related to serotonergic dysfunction"

13.00 – 14.10 Lunch break

14.10 – 14.55 **Edward Lemke** "Decoding molecular plasticity from single molecules to large assemblies"

14.55 – 15.40 **Andre Zeug** "How to visualize protein's function: Quantitative molecular microscopy approaches"

15.40 – 16.00 **Alexander Wirth** " Cellular functions of Cdc42 are dependent on lipid modifications

16.00 – 16.20 Coffee break

16.20 – 17.05 **Yury Prokazov** "From large field microscopy to single photon counting"

17.05 – 17.45 **Andrew Woehler** "Mulitsensor FRET microscopy: towards spatiotemporal correlation of intracellular signaling processes"

17.45 - 18.05 **Michał Stawarski** "FRET-based MMP-9 activity sensor"

19.00 – **Get-together party**

Friday, May 30th

9.00 – 9.20 **Antonina Khoruzhenko** "mTOR association with nucleoli and intermediate filaments in human normal and breast cancer cells"

9.20 – 9.40 **Katarzyna Rojek** "Expression and function of Angiomotin family of proteins in the brain"

9.40 – 10.00 **Kamil Parobczak** “Arc functional neighborhood in the nucleus”
10.00 – 10.20 **Agnieszka Walczak** “Spatial repositioning of the Bdnf gene upon seizures”

10.20 – 11.05 **Błażej Ruszczycki** “Before practicals: computational methods in (neuro)biological image analysis”

11.05 – 11.25 Coffee break
11.25 – 13.25 **Practicals**
13.25 – 14.30 Lunch break
14.30 – 18.30 **Practicals**

18.30 – **Wine and cheese at the posters**

Saturday, May 31st

9.00 – 11.00 **Practicals**
11.00 – 11.30 Coffee break
11.30 – 13.30 **Practicals**
13.30 - Lunch

Abstracts:

Full time lectures

Ireneusz Grulkowski

"Multidimensional interferometric imaging and metrology in biomedicine – from microscopy to mesoscopy"

Optical imaging is an emerging technology which offers unprecedented ability to visualize the internal structure of the objects even with submicrometer resolution. Due to extensive research, rapid advances resulted in developing new imaging modalities. Optical imaging has found numerous applications especially in biomedicine, where it offers potential to differentiate tissues without exogenous contrast or demonstrates ability to probe physiological properties of tissues. With these features, optical imaging technologies are becoming powerful clinical tools for non-invasive and objective diagnosis, guided treatment and monitoring therapies.Optical Coherence Tomography (OCT) and its microscopic version (Optical Coherence Microscopy, OCM) belong to rapidly developing methods that revolutionized medical diagnostics especially in ophthalmology. OCT is analogous to ultrasound B-mode imaging, using light rather than acoustical waves, and can perform micron scale imaging of microstructure in biological tissues in situ and in real time. Cross-sectional and three-dimensional images are generated by scanning an optical beam over the tissue and measuring the echo time delay and intensity of backscattered light. In this presentation, we will introduce the principles of OCT imaging and demonstrate numerous applications in biology and medicine. Current developments of imaging approaches will be shown that enable multidimensional OCT imaging of biological objects at different spatial and temporal scales.

Yannick Schwab

"Correlative Light and Electron Microscopy: taking snapshots of the living at the ultrastructural level"

Abstract

Our work is focused on the development of methods that enable high-resolution snapshots of dynamic events in cells and small model organisms. To achieve that, correlating light and electron microscopy is a powerful solution, because it combines functional imaging (fluorescent microscopy, time lapse imaging) with an enhanced ultrastructural readout (electron microscopy).

Jonas Ries

“Superresolution Microscopy of Protein Structures in Situ”

Abstract

We developed a method to use any GFP-tagged construct in single-molecule super-resolution microscopy. By targeting GFP with small, high-affinity antibodies coupled to organic dyes, we achieved nanometer spatial resolution and minimal linkage error when analyzing microtubules, living neurons and yeast cells. We show that in combination with libraries encoding GFP-tagged proteins, virtually any known protein can immediately be used in super-resolution microscopy and that simplified labeling schemes allow high-throughput super-resolution imaging.

Yury Prokazov

“From large field microscopy to single photon counting

Abstract

Exploring dynamic molecular processes within living cells requires high resolution imaging techniques that allow sensitive measurements in living cells at physiological conditions and correlate the functional imaging with the underlying morphological substrate.

In recent years much progress has been achieved in various fields of microscopy, fluoresence sample labeling and image processing. Among them are large field scans through small organisms (e.g. clarity), genetically-encoded dyes that allow live-cell imaging, correlated light- and electron microscopic approaches, super-resolution fluoresence microscopy (e.g. STED, PALM/STORM), special illumination techniques (light sheet) and methods to extract functional information from the fluorescence decay process (e.g. Fluorescence Lifetime Imaging (FLIM)).

Here, we present an overview of recent projects carried out within our Combinatorial NeuroImaging Core Facility at LIN (http://cni.ifn-magdeburg.de/de/index.html) to study synaptic plasticity in the nervous and immune system. Particular focus will be laid on high resolution imaging of synaptic proteins [1] and low light wide-field fluorescence lifetime imaging microscopy (FLIM) [2] to monitor dynamics of metabolic states (by NADH autofluorescence) [3] or fluorophore-labeled biosensors [4].

For FLIM a large number of detection techniques are employed nowadays. The most widespread detectors, implementing single photon counting based approach, are: photon multiplier tubes (PMTs) or single photon avalanche diodes (SPADs), mainly used in

combination with scanning microscopes and micro channel plate based PMTs (MCP-PMTs) used for wide-field applications. In addition, there are a number of integrating (opposite to counting) detectors: gated CCD cameras, streak cameras and conventional photodiodes.

In order to monitor inter- and intramolecular interactions of macromolecular complexes and to visualize living cells under low-light conditions for long time, our group has developed an ultrasensitve, position- and time-resolving wide-field camera system based on multi-channel plate electron amplification (gain up to 107) [5]. The detector has a sensitive area of 490 mm2 (i.e. diameter of 25 mm) and the electronic interface allows time- and space correlated single photon counting (TSCSPC) with a count rate of 600 kHz. The positional accuracy (i.e. the space resolution) of the camera is <20 µm (at the detector) and the time resolution is <50 ps. The camera can be connected easily to any microscope via c-mount, but needs a pulsed laser source for acquiring time information. The principle of the detector system and recent applications will be presented.

[1] Hradsky J, Mikhaylova M, Karpova A, Kreutz MR, Zuschratter W (2013) Super-resolution microscopy of the neuronal calcium-binding proteins calneuron-1 and caldendrin. Methods Mol Biol 963:147-169.

[2] Vitali M, Picazo F, Prokazov Y, Duci A, Turbin E, Gotze C, Llopis J, Hartig R, Visser AJ, Zuschratter W (2011) Wide-Field Multi-Parameter FLIM: long-term minimal invasive observation of proteins in living cells. PLoS One 6(2):e15820.

[3] Weber A, Prokazov Y, Zuschratter W, Hauser MJ (2012) Desynchronisation of glycolytic oscillations in yeast cell populations. PLoS One 7(9):e43276.

[4] Stirnweiss A, Hartig R, Gieseler S, Lindquist JA, Reichardt P, Philipsen L, Simeoni L, Poltorak M, Merten C, Zuschratter W, Prokazov Y, Paster W, Stockinger H, Harder T, Gunzer M, Schraven B (2013) T cell activation results in conformational changes in the SRC family kinase lck to induce its activation. Sci Signal 6(263):ra13.

[5] Hartig R, Prokazov Y, Turbin E, Zuschratter W (2014) Wide-Field Fluorescence Lifetime Imaging with Multi-anode Detectors. Methods Mol Biol 1076:457-480.

Andrew Woehler

Mulitsensor FRET microscopy: towards spatiotemporal correlation of intracellular signaling processes

Abstract

We have developed a novel method for multi-color spectral FRET analysis which is used to study a system of three independent FRET-based molecular sensors composed of the combinations of only three fluorescent proteins. This method is made possible by a novel

routine for computing the 3-D excitation/emission spectral fingerprint of FRET from reference measurements of the donor and acceptor alone. By unmixing the 3D spectrum of the FRET sample, the total relative concentrations of the fluorophores and their scaled FRET efficiencies are directly measured, from which apparent FRET efficiencies can be computed. If the FRET sample is composed of intramolecular FRET sensors it is possible to determine the total relative concentration of the sensors and then estimate absolute FRET efficiency of each sensor. Using multiple tandem constructs with fixed FRET efficiency as well as FRET-based calcium sensors with novel fluorescent protein combinations we demonstrate that the computed FRET efficiencies are accurate and changes in these quantities occur without crosstalk. We provide an example of this method's potential by demonstrating simultaneous imaging of spatially colocalized changes in [Ca(2+)], [cAMP], and PKA activity.

Evgeni Ponimaskin

“Regulation of receptor functions by heterodimerization of serotonin receptors”

Abstract

G protein-coupled receptors (GPCRs) participate in the regulation of many cellular processes and, therefore, represent key targets for pharmacological treatment. The existence of GPCR homo- and heterodimers has become generally accepted, and a growing body of evidence points to the functional importance of oligomeric complexes for the receptor trafficking, receptor activation, and G protein coupling in native tissues. Quantitative molecular microscopy is becoming more and more important to investigate such receptor-receptor interaction in their native environments. Förster resonance energy transfer (FRET) is thereby utilized to aim at investigating the interaction of molecules at distances beyond diffraction-limited spatial resolution. The exact determination of the FRET signals, which are often only fractions of the fluorescence signals, requires extensive experimental effort. Moreover, the correct interpretation of FRET measurements as well as FRET data-based modeling represents an essential challenge in microscopy and biophysics. I will present and discuss variety of acquisition protocols and models based on "linear unmixing FRET" (lux-FRET) to investigate receptor-receptor interaction in living cells with high spatial and temporal resolution. I will show how to apply lux-FRET in spectroscopic and different imaging devices, based either on spectral detection or on filter cubes. I will focus on detailed description for FRET measurements and analyses based on sophisticated acquisition procedures according to different experimental setups and also provide several examples of biological applications.

Andre Zeug

“How to visualize protein’s function: Quantitative molecular microscopy approaches”

Abstract

Förster resonance energy transfer (FRET) has become an important tool for analyzing different aspects of interactions among biological macromolecules in their native environments. FRET analysis has also been successfully applied to study the spatiotemporal regulation of various cellular processes using genetically encoded FRET-based biosensors. A variety of procedures have been described for measuring FRET efficiency or the relative abundance of donor-acceptor complexes, based on analysis of the donor fluorescence lifetime or the spectrally resolved fluorescence intensity. The latter methods are preferable if one wants to not only quantify the apparent FRET efficiencies but also calculate donor-acceptor stoichiometry and observe fast dynamic changes in the interactions among donor and acceptor molecules in live cells. I focus on a comparison of the available intensity-based approaches used to measure FRET.

Edward Lemke

“Decoding molecular plasticity from single molecules to large assemblies”

The growing demands of advanced fluorescence and super-resolution microscopy benefit from the development of small and highly photostable fluorescent probes. Techniques developed to expand the genetic code permit the residue-specific encoding of unnatural amino acids (UAAs) armed with novel clickable chemical handles into proteins in living cells. Here we present the design of new UAAs bearing strained alkene side chains that have improved biocompatibility and stability for the attachment of tetrazine-functionalized organic dyes by the inverse-electron-demand Diels-Alder cycloaddition (SPIEDAC). Furthermore, we fine-tuned the SPIEDAC click reaction to obtain an orthogonal variant for rapid protein labeling which we termed selectivity enhanced (se) SPIEDAC. seSPIEDAC and SPIEDAC were combined for the rapid labeling of live mammalian cells with two different fluorescent probes. We demonstrate the strength of our method by visualizing insulin receptors (IRs) and virus-like particles (VLPs) with dual-color super-resolution microscopy.

Short talks:

Matthia Winter-Karreman

Lights Will Guide You: Tracking Invasive Tumor Cells with Correlative Intravital Fluorescence Microscopy and Electron Microscopy

Metastasis is the main cause of cancer mortality, and an important target for therapeutics. The major events in cancer metastasis involve detachment of cells from the tumor (invasion), intrusion into vessels (intravasation), circulation, exit from the vessel, and secondary site colonization. There is currently no method allowing correlation of tumor cell behavior to its most detailed architecture. We aim to image the ultrastructural alterations to the cancer cells and their microenvironment that enable their invasion and intravasation. Hereto we employ a correlative approach, combining intravital imaging and electron microscopy, allowing capturing these rare events.

We developed a xenograft model where fluorescent cancer cells are injected subcutaneously into the mouse ear and subsequently imaged using two-photon-excitation microscopy. Near-infrared branding (NIRB) is used to mark this imaged area: using laser irradiation, a square is drawn at the skin level, just above the region of interest (ROI), allowing full preservation of this area. The distance from the NIRB square to the ROI is then used as a guide to locate the ROI in a later stage.

Herein, we show that we successfully retrieve the ROI from several samples. The concerted usage of the positioning of the NIRB square and structural features in the sample as landmarks enables determining and approaching the position of the ROI while serial sectioning through the specimen. Finally, the cancer cells can be imaged at high resolution and detailed analysis of the cells' ultrastructure.

In conclusion, we show here how we could track an interesting cellular behavior imaged in vivo using a correlative imaging approach. The tedious and time-consuming process represents a first step in our work to develop an automated workflow for high-throughput, high resolution, 3D analysis of the dynamic steps of cancer metastasis.

Adriana Magalska

Loss of neuronal 3D chromatin organization causes transcriptional and behavioral deficits related to serotonergic dysfunction

interior of the neuronal cell nucleus is a highly organized 3-dimensional (3D) structure in which regions of the genome that are millions of bases apart participate in specialized sub-

structures with dedicated functions. To investigate neuronal chromatin organization and dynamics in vivo, we generated bitransgenic mice that express histone GFP-tagged H2B in principal neurons of the forebrain. Surprisingly, the expression of this chimeric histone in mature neurons causes chromocenter declustering and disrupts the association of heterochromatin with the nuclear lamina. The loss of these structures does not affect neuronal viability but is associated with specific transcriptional and behavioral deficits related to serotonergic dysfunction. Overall, our results demonstrate that the 3D-organization of chromatin in the neuronal nucleus supports an additional level of epigenetic regulation of gene expression that critically influences neuronalfunction and indicate that some loci associated with neuropsychiatric disorders may beparticularly sensitive to changes in chromatin architecture.

Alexander Wirth

Cellular functions of Cdc42 are dependent on lipid modifications

Cdc42 (cell division cycle 42) is a member of the Rho GTPase family which regulates a variety of cellular activities by controlling actin cytoskeleton and gene expression. Cdc42 is expressed in the form of two splice variants. The canonical Cdc42 isoform is prenylated (Cdc42-prenyl), whereas the brainspecific isoform can be palmitoylated (Cdc42-palm). In the present study we have demonstrated palmitoylation of endogenous Cdc42 in rodent and human brains and identified Cys188 and Cys189 as acylation sites of Cdc42-palm. Moreover, we have shown that Cys188 can also be prenylated. Analysis of acylation-deficient mutants revealed that lipidation of Cys188 is essential for proper membrane binding of Cdc42-palm as well as for Cdc42-mediated regulation of gene transcription and induction of densely packed filopodia in neuroblastoma cells. We also found that Cdc42-prenyl is a dominant splice variant in a wide range of commonly used cell lines as well as in the cerebellum, whereas Cdc42-palm is the main Cdc42 isoform in hippocampus, where it is critically involved in the formation of dendritic filopodia and spines. Replacement of endogenous Cdc42 by its acylation-deficient mutants revealed the importance of Cdc42-palm lipidation for its morphogenic and synaptogenic effects in neurons. These findings demonstrate that dual lipidation of Cdc42-palm represents an important regulator of morphogenic signalling in hippocampal neurons.

Michał Stawarski

“FRET-based MMP-9 activity sensor”

A genetically encoded Förster Resonance Energy Transfer (FRET)-based biosensor that continuously monitors matrix metalloproteinase 9 (MMP-9) activity was developed. MMP-9 is an extracellularly acting endopeptidase with a prominent role in development, learning and memory, cancer metastasis, and stroke. To assess the biological function of the protease, determining the precise kinetics and localization of MMP-9 activity is required. The nontoxic,

genetically encoded FRET biosensor presented herein is anchored in the cellular membrane and thus provides an important advantage over currently employed probes. The biosensor allows the study of the proteolytic activity of MMP-9 with high temporal and subcellular resolution at the precise region of MMP-9 action on the cell. The applicability of the biosensor both in vitro and in living cells was demonstrated by ratiometrically analyzing the cleavage of the biosensor by a purified auto-activating mutant of MMP-9 and endogenously secreted protease in cultured tumor and neuronal cells. The precise kinetics of endogenous MMP-9 activity was measured, which demonstrates in a straight-forward manner the applicability of the biosensor concept.

Antonina Kohoruzhenko

mTOR association with nucleoli and intermediate filaments in human normal and breast cancer cells

Kinase mTOR is one of the main links in signal transduction from variety of growth factors and hormones into the cell. mTOR participates in the regulation of protein synthesis, cell growth, survival and proliferation. Earlier it was demonstrated significant overactivation of mTOR in numerous of malignant neoplasms. But there are controversial data concerning its subcellular localization. So, subcellular localization of this kinase was detected in breast cancer and normal tissue, MCF-7 cell monolayer and 3D cultures. Antibodies, generated to C-, N-terminus and central sites of mTOR served as a tool for such a determination. Detection of mTOR subcellular distribution with antibodies to C-terminal region of mTOR revealed predominantly diffuse cytoplasmic localization in all cases. Application of the monoclonal antibodies to the central site of the kinase revealed its additional nucleolar localization in MCF-7 cells. Nucleolar localization of mTOR kinase was observed in range of breast cancer tissue in contrast to surrounding normal tissue. Detection of mTOR localization with anti N-terminal antibodies revealed a stained network in cells. Confocal microscopy analysis, co-immunoprecipitation and PLA showed strong co-localization of mTOR and intermediate filaments (cytokeratins) in MCF-7 cells. Also such co-localization of mTOR and cytokeratins was detected at the histological sections of normal and malignant breast tissue. So, for the first time the nucleolar localization of mTOR was demonstrated. Moreover, it was revealed the co-localization of mTOR kinase and intermediate filaments.

Katarzyna Rojek

“Expression and function of Angiomotin family of proteins in the brain”

Proper function of synaptic connections is important for the transmission of information in the central and peripheral nervous systems (CNS and PNS). The molecular mechanisms underlying synaptic remodeling are still poorly understood. We have recently identified the scaffold protein Angiomotin-like-2 (Amotl2), as a potential regulator of neuromuscular junction (NMJ) plasticity. Interestingly, majority of the machinery that specifically regulates

NMJ remodeling is also implicated in the plasticity of synapses in the brain. Therefore, we investigated the expression of angiomotin-like-2 (Amotl2) in the CNS. Our analysis also included the closely related proteins angiomotin (Amot) and angiomotin-like-1 (Amotl1), which together with Amotl2 constitute a family of proteins called angiomotins. We demonstrated that all three angiomotins are widely expressed in the brain. In cultured rat hippocampal neurons and mouse brain slices Amotl2 and Amotl1 localize to the synaptic compartment, whereas Amot was distributed in neurites with the more predominant expression in mature axons. Thus, our experiments identified a novel group of proteins that may regulate synaptogenesis both in the CNS and PNS and highlight parallels between synapses in both systems. We are currently performing knock-down experiments and generating conditional knockout mice to study the function of Angiomotins in the CNS and PNS. Results from our preliminary experiments using RNAi suggest that Amot plays a role in neurites outgrowth and Amotl2 is involved in synaptogenesis.

Kamil Parobczak

"Arc functional neighborhood in the nucleus"

The immediate early gene protein Arc is required for long-term memory formation and multiple forms of activity-dependent synaptic plasticity. At synapses, Arc enhances internalization of AMPA-type glutamate receptors in LTD, while Arc function in LTP is coupled to regulation of actin cytoskeletal dynamics. The role of Arc in the nucleus is little understood. Recently, Arc was reported to inhibit transcription of glutamate receptors through a mechanisms involving Arc binding to promyelocytic leukemia (PML) protein. Here, we performed structural, functional and biochemical analysis to identify Arc`s nuclear interactome in vivo. Confocal microscopy showed that Arc occupies internal parts of the nucleus, closely associated with hnRNPs. Electron microscopy further revealed labeling at the peripheral areas of chromatin. Arc pulldown experiments performed in nuclear fractions suggest that Arc interacts with component of the splicing machinery. Collectively, our data suggest that nuclear Arc is involved in pre-mRNA processing. These data underscore multiple roles for Arc protein carried out within distinct subcellular domains.

Agnieszka Walczak

"Spatial repositioning of the Bdnf gene upon seizures"

Studies in cultured cells have demonstrated the existence of higher-order epigenetic mechanisms, determining the relationship between expression of the gene and its position within the cell nucleus. It is unknown, whether such mechanisms operate in postmitotic, highly differentiated cell types, such as neurons in vivo. Accordingly, we examined whether the intranuclear positions of Bdnf and Trkb genes, encoding the major neurotrophin and its receptor respectively, change as a result of neuronal activity, and what functional consequences such movements may have. In a rat model of massive neuronal activation upon

kainate-induced seizures we found that elevated neuronal expression of Bdnf is associated with its detachment from the nuclear lamina, and translocation toward the nucleus center. In contrast, the position of stably expressed Trkb remains unchanged after seizures. Our study demonstrates that activation-dependent architectural remodeling of the neuronal cell nucleus in vivo contributes to activity-dependent changes in gene expression in the brain.