International Symposium

Analysis of Biomolecular Machines in the Nanometer Range

September 20th – 23th, 2009

Rosensäle, Jena, Germany

Abstractbook
Program


PHOTONICS
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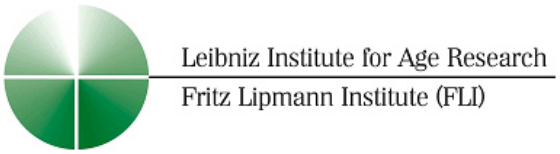
Light for Life Sciences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International Symposium *Analysis of Biomolecular Machines in the Nanometer Range*

Program Overview

Sunday 20.9.2009	Monday 21.9.2009	Tuesday 22.9.2009	Wednesday 23.9.2009	Thursday 24.9.2009
	Session 1 - S. Diekmann	Session 3 – C. Cremer	Session 4 – P. Hemmerich	
	9:00 h C. Cremer 9:40 h R. Heintzmann 10:20 h D. Lamb 11 h Coffee break 11:30 h D. Baddeley 12:10 h A. Naber 12:40 h U. Birk	9:00 h T. Cremer 9:40 h K. Rippe 10:20 h S. Diekmann 11 h Coffee break 11:30 h D. Gerlich	9:00 h R. van Driel 9:40 h D. Bazett-Jones 10:20 h A. von Mikecz 11 h Coffee break 11:30 h U. Kubitscheck 12:10 h J. Fitter	Departure
	13:00 h – 14:00 h Lunch	12:30h – 13:30 h Lunch	13:00 h – 14:00 h Lunch	
Arrival	Poster Session 14:00 h – 15:30 h	Posters free afternoon	Posters	
16 – 19 h Check in	Session 2 – A. von Mikecz	optional 14 h Goethes color theory (C. Cremer)	Session 5 – R. Heintzmann	
Optional: 17 – 18 h General guided city tour	15:30 h C. Cardoso 16:10 h J. Langowski 16:50 h H. Leonhardt 17:30 h J. McNally	15 h Optical Museum 16:30-18:30 h special guided city tour “Jena 1800”	15:30 h J. Fandrey 16:10 h A. Egner 16:50 h G. Gerisch	
19 h welcome	18:30 h – 19:30 h Dinner - Jena Market place		18:00 h Closing Lecture J. Lippincott-Schwartz	
20 h Opening Lecture E. Gratton	20 h Evening lecture P. Carlton		20:00 h Dinner - Fuchsturm	

Program

Sunday, September 20th, 2009

- 16:00 – 19:00 — *Check in*
  Set up Posters

Optional

- 17:00 – 18:00 — *General guided city tour Jena*
  Meeting point: Reception Rosensaele

- 19:00 — Welcome

Opening Lecture

- 20:00 — Enrico Gratton
  Imaging nanometer structures using modulation tracking

Monday, September 21st, 2009

Session I - S. Diekmann

- 09:00 – 09:40 — C. Cremer
  Lightoptical Nanoimaging of Cellular Structures
- 09:40 – 10:20 — R. Heintzmann
  Microscope Resolution enhancement with Image Inversion Interferometry
- 10:20 – 11:00 — D. Lamb
  Single Virus Tracking: The kinetics of HIV Assembly and New Methods for following Individual Biomolecules
- *Coffee Break*
- 11:30 – 12:10 — D. Baddeley
  Routine imaging with 30nm optical resolution allows novel insight into cardiac calcium signalling
- 12:10 — A. Naber
  Near-field optical study of protein transport kinetics at a single nuclear pore
- 12:40 — U. Birk
  Optical Projection Tomography (OPT) for In-Vivo Applications
- 13:00 – 14:00 — *Lunch*
- 14:00 – 15:30 — **Poster session**

Session II - A. von Mikecz

- 15:30 – 16:10 — C. Cardoso
  Chromatin labeling, accessibility and mobility
- 16:10 – 16:50 — J. Langowski
  Nucleosome structural variations characterized by single molecule FRET
- 16:50 – 17:30 — H. Leonhardt
  Studying nuclear structure and function with 3D structured illumination microscopy (3D-SIM) and fluorescent nanobodies
- 17:30 — J. McNally
  Kinetic Modeling of the Dynamic Nucleus: What do we really know?
- 18:30– 19:30 — Dinner - Jena Market place

Evening lecture

- 20:00 — P. Carlton
  Widefield microscopy with high spatial and temporal resolution

Tuesday, September 22nd, 2009

Session III - C. Cremer

- 09:00 – 09:40 — T. Cremer
  Nuclear architecture studied by microscopy: current state and future perspectives
- 09:40 – 10:20 — K. Rippe
  PML Nuclear Bodies in Telomerase independent Lengthening of Telomeres
- 10:20 – 11:00 — S. Diekmann
  The human core and inner NAC kinetochore proteins: dynamics and architecture of their complex
- *Coffee Break*
- 11:30 — D. Gerlich
  Automated live imaging to study human cell division
- 12:30 – 13:30 — Lunch
- **Posters/free afternoon**

Optional

- 14:00 — Goethes Color Theory
  (C. Cremer)
- 15:00 — *Optical Museum*
- 16:30 – 18:30 — *Special guided city tour "Jena at 1800"*

Wednesday, September 23rd, 2009

Session IV - P. Hemmerich

- 09:00 – 09:40 — R. van Driel
  In vivo assembly and functioning of a chromatin-associated protein complex: the chroreography of DNA repair proteins as a paradigm
- 09:40 – 10:20 — D. Bazett-Jones
  Electron Spectroscopic Imaging of Chromatin Reveals Significant Reorganization of the Genome Associated with induced Pluripotency
- 10:20 – 11:00 — A. von Mikecz
  Multiscale analysis of nanoparticle dynamics and interactions in living human cells
- *Coffee Break*
- 11:30 – 12:10 — U. Kubitscheck
  Intranuclear trafficking of native mRNA
- 12:10 — J. Fitter
  Translational diffusion and interaction of a photoreceptor and its cognate transducer observed in giant unilamellar vesicles by using dual-focus FCS
- 13:00 – 14:00 — *Lunch*
- 14:00 — **Posters**

Session V - R. Heintzmann

- 15:30 – 16:10 — J. Fandrey
  Molecular views on cellular oxygen sensing
- 16:10 - 16:50 — A. Egner
  Developments in Fluorescence Nanoscopy
- 16:50 — G. Gerisch
  Self-organization of actin waves and the shaping of a phagocytic cup

Closing Lecture

- 18:00 — J. Lippincott-Schwartz
  Advances In Super-resolution Imaging Technologies
- 20:00 — *Dinner - Fuchsturm*

Imaging nanometer structures using modulation tracking

Enrico Gratton, Luca Lanzano' and Peter Fwu

Laboratory for Fluorescence Dynamics, University of California, Irvine

We describe a new modality of imaging nanostructures using modulation tracking based on optical feedback. The method uses a variant of circular tracking that we have developed some years ago for tracking particles and single molecules in cells. Since the method does not use the diffraction properties of light, we are not breaking the diffraction limit. We will show nanometer images of microvilli in living cells. The microvilli have a diameter of approximately 100 nm and length of several microns. The method allows the observation of patches of fluorescently labeled protein on the microvilli surface and the diffusion of fluorescent proteins along the microvilli membranes.

Lightoptical Nanoimaging of Cellular Structures

C.Cremer

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For a better understanding of the implications of nanostructure for the control of cellular function, it is necessary to improve methods of lightoptical resolution beyond the possibilities of conventional epifluorescence microscopy (optical resolution about 200 nm laterally, 600 nm axially). Here, we report on new approaches of lightoptical 'nanoimaging' using methods of Spatially Modulated Illumination (SMI) and of "Spectral Precision Distance/Position Determination Microscopy (SPDM) with Physically Modifiable Fluorochromes" ($SPDM_{Phymod}$). While SMI Microscopy allowed to measure sizes of individual fluorescent complexes down to the range of few tens of nm in the direction of the optical axis, $SPDM_{Phymod}$ made it possible to analyse the lateral distribution of single molecules (membrane proteins, nuclear proteins, DNA sequences) at the macromolecular optical resolution level. A specific feature of $SPDM_{Phymod}$ is the use of conventional fluorophores such as Alexa dyes, GFP, YFP, mRFP. SPDM analysis of membrane protein distribution was performed with an optical lateral resolution down to the 10 nm regime (~ 1/50 of the exciting wavelength); combining SPDM and SMI Microscopy allowed an optical 3D resolution of 40 – 50 nm.
The intranuclear spatial location of two species of single molecules in human cells was determined with a precision down to a few nm and up to a density of ca. 1000 molecules/μm^2; single molecule distances down to 15 – 30 nm were nanoscopically resolved in optical sections of about 600 nm thickness. A quantitative analysis revealed characteristic spatial distribution patterns not observable by conventional epifluorescence microscopy. In other experiments, nanoimaging of specific FISH labeled chromatin regions was performed.

*J. Reymann et al. (2008) High precision structural analysis of subnuclear complexes in fixed and live cells via Spatially Modulated Illumination (SMI) microscopy. Chromosome Research **16**: 367 – 382.*

*P.Lemmer et al. (2008) SPDM – Light Microscopy with Single Molecule Resolution at the Nanoscale. Applied Physics B **93**: 1-12.*

*R. Kaufmann et al. (2009) SPDM – Single Molecule Superresolution of Cellular Nanostructures. (2009). Proc. SPIE **7185**: 71850J1 – 71850J-19.*

M. Gunkel et al. (2009) Dual color localization microscopy of cellular nanostructures. Biotechnology J. **4**: 927 – 938.

*P. Lemmer et al.(2009)Using conventional fluorescent markers for far-field fluorescence localization nanoscopy allows resolution in the 10-nm range. J. of Microscopy **235**: 163 – 171.*

Microscope Resolution enhancement with Image Inversion Interferometry

Kai Wicker, Simon Sindbert, Rainer Heintzmann

We present experimental results on image inversion interferometric setups with significant potential to increase efficiency and resolution in fluorescence confocal microscopy. Results agree with theory in terms of a reduction of FWHM. The basic idea was described previously: The emitted image signal is sent through an interferometer in which one of the two paths inverts (rotates by 180 degrees) its image information. Constructive interference is only possible, if the emitter is right on the optic axis as both mirror images are then ideally identical. This forms the basis of the resolution improvement in a confocal setup. The designs have significant potential to increase efficiency and resolution in fluorescence confocal microscopy.

Single Virus Tracking: The kinetics of HIV Assembly and New Methods for following Individual Biomolecules.

Don C. Lamb

Using single virus tracking (SVT), individual viruses can be followed with nanometer accuracy as they infect, reproduce and are released from living cells. Hence, we can investigate the dynamics of different portions of the life cycle of viruses in unprecedented detail. In the beginning of my talk, I will introduce the method of SVT. We have then applied SVT to investigate the assembly and release of HIV-1. Using a combination of wide-field and total internal reflection fluorescence microscopy, we have investigated assembly of fluorescently labeled HIV-1 at the plasma membrane of living cells with high time resolution. Gag assembled into discrete clusters corresponding to single virions. Assembly occurred within approximately 25 minutes and three phases were observed. In phase I, Gag molecules are recruited to the budding site within the first 10 minutes. In Phase II, a plateau in fluorescence intensity is observed with no exchange of Gag protein. Phase II is followed by a decay in fluorescence intensity, referred to as phase III. This decay, in some cases, corresponds to the release of a virion. The time scale from the onset of assembly to release of extracellular particles was measured to be ~1,500+/-700 s.

In the last portion of my talk, I will focus on a novel SVT microscope we have developed for tracking individual viruses in 3D in real time. In this microscope, the laser beam is orbited about the particle and from the phase and modulation of the fluorescence signal, the position determined in two dimensions. For the third dimension, two confocal planes, immediately below and above the particle, are monitored simultaneously. The position of the tracked particle is determined in 3D in real time and the orbit of the laser beam is recentered on the particle using a feedback loop. In addition to orbital tracking, a wide-field detection system is incorporated into the microscope. Hence, the local environment of the tracked molecule can be simultaneously observed. We have applied this microscope to measure the motion of artificial viruses and nanoparticles in live cells.

Routine imaging with 30nm optical resolution allows novel insight into cardiac calcium signalling

David Baddeley

Recently developed super-resolution techniques based on the localisation of single fluorescent molecules have allowed far field optical imaging with an approximately 10-fold improvement in resolution. When initially introduced, localisation microscopy relied on unusual fluorophores which were not trivial to obtain. Within the last year it has become apparent that virtually any fluorophore can be induced to demonstrate the required switching characteristics given a suitable chemical environment and illumination conditions.

I will present several of our advances, spanning the regimes of image acquisition, through to data analysis and processing which together have made localisation microscopy comparable in terms of ease of use and throughput to standard confocal microscopy. These advances have allowed us to characterise the distribution of 'ryanodine receptors' which are intracellular Ca^{2+} channels in heart muscle cells. The receptors are arranged in clusters below the surface membrane where they form ‘peripheral couplings’ or ‘couplons’. Surprisingly we have found that these 'couplons' exhibit a wide range of sizes and contain almost an order of magnitude fewer RyRs on average than was previously estimated using electron microscopy. The large N numbers (3576 clusters) that can be sampled using light microscopy also enable us to analyse the shape of the size distribution and to propose a stochastic self-assembly model of RyR cluster formation.

Near-field optical study of protein transport kinetics at a single nuclear pore

N. Neuberth,[a)] M. Herrmann,[b)] J. Pérez,[a)] J. Wissler,[a)] D. Gradl,[b)] and A.Naber [a)]

a) Institut für Angewandte Physik, Universität Karlsruhe (TH), Germany
b) Zoologisches Institut II, Universität Karlsruhe (TH), Germany

Over the last years the demand for high-resolving imaging techniques capable of observing kinetic biological processes on a nanometric scale has grown immensely. In particular far-field optical fluorescence techniques have proven most useful due to advanced preparation methods and unmatched sensitivity. Using common techniques, however, a localization of single molecules on a nano-scale can be accomplished only on the expense of a largely diminished temporal resolution in the range of milliseconds or seconds. In contrast, near-field scanning optical microscopy (SNOM) is in principal capable of achieving a spatial resolution down to 10-30 nm without compromising time resolution. In biology, SNOM has been used mainly on dried samples but progress regarding probe-sample distance control recently enabled also imaging of unfixed membranes in liquid [1]. However, an investigation of transport processes was not feasible so far since imaging the soft membrane still required a solid support.
We now solved a major obstacle of observing translocation events by establishing a novel distance control based on the weak elastic response of a freely suspended membrane patch [2]. The “passive” distance control enabled us to reliably image the nuclear pore complexes (NPCs) embedded in a native nuclear envelope (NE) at an optical resolution of typically 30-50 nm. NPCs are highly differentiated macromolecular assemblies of ring-like structure that regulate the exchange of molecules between nucleus and cytoplasm. As the transport channel of the NPCs was no longer obstructed by a support, the translocation of fluorescence-labeled proteins (NTF2) could be observed by placing the SNOM aperture probe over a NPC and detecting the fluorescence of NTF2 as a function of time. An evanescent field at the aperture with a decay length of 10-25 nm produced a steep intensity gradient along the axis of the NPC, so that the proteins generated strong fluorescence fluctuations by traveling up and down the transport channel. By analyzing the fluctuations using fluorescence correlation spectroscopy (FCS) we obtained previously inaccessible kinetic information about the transport process through a NPC and thereby provided new insights into the still unknown mechanism of the NPC as gated permeability barrier [2].

[1] C. Höppener, J. P. Siebrasse, R. Peters, U. Kubitscheck, and A. Naber, “High-Resolution Near-Field Optical Imaging of Single Nuclear Pore Complexes under Physiological Conditions”, Biophys. J. 88, 3681-8 (2005).
[2] M. Herrmann, N. Neuberth, J. Wissler, J. Pérez, D. Gradl, and A. Naber, "Near-Field Optical Study of Protein Transport Kinetics at a Single Nuclear Pore", *Nano Lett.* **9**, 3330-3336 (2009).

Optical Projection Tomography (OPT) for In-Vivo Applications

Udo Birk[1], Matthias Rieckher[2], Ana Sarasa Renedo[1], Clio Mamalaki[2], Alex Darrell[1], Nektarios Tavernarakis[2], and Jorge Ripoll[1]

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Optical projection tomography is a recently developed technique used to acquire 3D microscopy data from specimens which are 1-10mm thick[1]. In previous work, the specimens were fixed and cleared in order to reduce scattering and attenuation; a process that makes in-vivo fluorescence OPT of the sample impossible. We present a novel OPT method for obtaining 3D images of both anatomy and fluorescent protein expression in-vivo and we apply this technique to several specimens e.g., for gene expression studies in *Caenorhabditis elegans*.
During testing of the OPT setup we performed a series of OPT experiments on *Drosophila melanogaster* at different stages of the development (larvae, pupa and adult) to target different expression patterns. After a redesign of the illumination and detection light paths, experiments on *Caenorhabditis elegans* (in-vivo) and on mouse spleen (ex-vivo, fixed and cleared) were performed showing the feasibility of the OPT setup for fluorescence imaging of expression patterns in these specimens. Novel software correction methods have been applied after the images were acquired in order to reduce the influence of object movement and thus to increase the resolution achievable. For larger specimens such as mouse spleen, selection of different objective lenses permits imaging with optimal magnifications and field of view.
We have successfully imaged GFP expression in live *C. elegans* and obtained 3D maps of the touch sensor related neuronal network. These 3D maps can be complemented by 3D morphology (absorption) reconstructions unique to this imaging technique. The OPT setup can be used in white light transmission mode to study morphometry during the development stages delivering high (~1.5μm) spatial resolution not only for cleared specimens but also for living slightly opaque organisms. With the addition of fluorescence imaging OPT allows studies of fluorescence distribution and thus of gene expression patterns in vivo.
We believe this approach will prove useful for in vivo follow-up measurements of gene expression patterns also in mm-sized samples, traditionally unavailable when using imaging techniques such as confocal microscopy.

Acknowledgement: U. Birk gratefully acknowledges support by the EU project MEIF-CT-2006-041827.

References:

1. J. Sharpe et al. (2002) “Optical Projection Tomography as a Tool for 3D Microscopy and Gene Expression Studies”, Science 296.
2. H. Meyer et al. (2008) “Optical Projection Tomography for In-Vivo Imaging of Drosophila melanogaster”, Microsc. Analys. 22(5).

Chromatin labeling, accessibility and mobility

M. Cristina Cardoso

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Most cells of multicellular organisms contain identical genetic information but differ in their epigenetic information. We are interested on how genetic and epigenetic information is replicated every time a cell divides and how the epigenetic information is accessed and "translated" to define specific cell types with specific sets of active and inactive genes, collectively called the epigenome.

It is unclear whether and how changes in the chromatin compaction state, in particular the heterochromatic state, affect the mobility of chromatin, its organizing proteins and the access of proteins to chromatin. To address these questions, we are using a combination of live-cell chromatin labels and high-speed single molecule tracking microscopy in living mammalian cells. Our results indicated that all nuclear subcompartments were easily and similarly accessible for an average-sized probe protein (streptavidin) and even condensed heterochromatin did neither exclude single molecules nor impede their passage. The only significant difference was a higher frequency of transient trappings in heterochromatin lasting though, only tens of milliseconds. The streptavidin molecules, however, did not accumulate in heterochromatin suggesting comparatively less free volume. Finally, we labeled different chromatin subsets by fluorescent nucleotide incorporation during DNA replication and tracked their mobility throughout the cell cycle by particle tracking. We will discuss our chromatin mobility data and its interdependency of nuclear metabolism and topology.

Nucleosome structural variations characterized by single molecule FRET

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The nucleosome has a central role in the compaction of genomic DNA and the control of DNA accessibility for transcription and replication. We studied the effect of DNA sequence and selective histone acetylation on the structure, stability and disassembly of the mononucleosomes. Quantitative single molecule FRET measurements between dyes attached to different parts of the nucleosome permitted us to detect the equilibrium between several subpopulations of reconstituted nucleosomes in solution. We obtained that the heterogeneity and stability of the samples are correlated with each other and influenced both by the DNA sequence and the histone acetylation. The path of the linker DNA is more sensitive to all studied effects than the DNA on the core. Intermediates of the disassembly pathway were identified and characterized.

Studying nuclear structure and function with 3D structured illumination microscopy (3D-SIM) and fluorescent nanobodies

Heinrich Leonhardt

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Fluorescence light microscopy allows multicolor visualization of cellular components with high specificity, but its utility has until recently been constrained by the intrinsic limit of spatial resolution. We applied three-dimensional structured illumination microscopy (3D-SIM) to circumvent this limit and to study the mammalian nucleus (*Science*, 320, 1332-6). By simultaneously imaging chromatin, nuclear lamina, and the nuclear pore complex (NPC), we observed several features that escape detection by conventional microscopy. We could resolve single NPCs that colocalized with channels in the lamin network and peripheral heterochromatin. We could differentially localize distinct NPC components and detect double-layered invaginations of the nuclear envelope in prophase as previously seen only by electron microscopy. Multicolor 3D-SIM opens new possibilities to analyze subcellular structures beyond the diffraction limit of the emitted light.
We have recently generated fluorescent, antigen-binding proteins, termed chromobodies, combining epitope-recognizing fragments with fluorescent proteins (*Nature Methods*, 3, 887-9). Unlike conventional antibodies these chromobodies can be expressed in living cells and used to target or trace epitopes in subcellular compartments. As chromobodies are soluble and active in the intracellular environment, they provide an optical readout for novel high content analyses and enable functional studies in vivo. These antigen-binding fragments can also be produced in *E. coli* and chemically functionalized. Despite being ten times smaller than conventional antibodies they efficiently bind their antigen and can be used for affinity purification and proteome analyses. This combination of biochemical and cellular analyses provides new insights into the structure and function of cells.

Kinetic Modeling of the Dynamic Nucleus: What do we really know?

James McNally

Live cell analysis by photobleaching microscopy has demonstrated that transcription factors, their co-factors and the polymerase itself are bound quite transiently at the promoters of many active genes. Quantitative analysis of these live cell data has yielded estimates of chromatin residence times for these different factors, plus qualitative predictions about how binding and assembly occur. However, all of these predictions rely on relatively new analysis methods. We have found that different methods produce very different answers. For example, quantitative estimates for residence times for the same factor can vary by as much as three orders of magnitude depending on the method of analysis. Beyond these numerical discrepancies, different methods of analysis can also yield very different qualitative predictions about the mechanisms of binding and assembly. I will present our efforts to develop more robust methods for a better understanding of the dynamics of transcription in living cells.

Widefield microscopy with high spatial and temporal resolution

Pete Carlton

We have developed an optical microscopy platform ("OMX") which implements 3D-Structured Illumination Microscopy (3D-SIM) imaging of fixed samples, as well as highly time-resolved 3D imaging of in vivo samples. I will discuss the theory of both imaging modalities as well as their recent applications: structural studies of the nuclear periphery and meiotic chromosomes in the case of 3D-SIM, and dynamic movement of yeast and C. elegans chromosome regions in the case of fast live imaging. Particular attention will be paid to the problem of recovering sufficient image information in the low light conditions required for viability under in vivo imaging.

Nuclear architecture studied by microscopy: current state and future perspectives

Thomas Cremer

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The proper storage, use and maintenance of genetic information depend on the complex interplay of epigenetic mechanisms with higher order nuclear organization. While epigenetics pursues the goal of decoding the chromatin language, including DNA methylation, histone modifications, nucleosome positioning and chromatin remodeling events, epigenomics has to fulfill the even more daunting task to understand the functional integration of these processes into nuclear architecture at large. Current models of nuclear architecture agree that chromosome territories are a basic feature of nuclear architecture, but they express decisively different opinions with respect to the organization and possible size range of chromatin loops, the dynamics of chromatin movements, gene interactions in *cis* and *trans*, the presence or absence of a distinct interchromatin compartment carrying splicing speckles and nuclear bodies, as well as the topography of RNA transcription, chromatin replication and DNA repair. These differences point to the huge gap of knowledge, which must still be bridged to achieve an integrated understanding of nuclear structure and function from the molecular level to the level of higher order organization. The recent development of light optical nanoscopy and 3D electron microscopy has provided new possibilities to study nuclear architecture at nanometer scale resolution with unprecedented detail and will help to put conflicting predictions of current models of nuclear architecture to decisive tests.

PML NUCLEAR BODIES IN TELOMERASE INDEPENDENT LENGTHENING OF TELOMERES

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Telomerase-negative tumor cells maintain their telomeres via an alternative lengthening of telomeres (ALT) mechanism. This process involves the association of telomeres with promyelocytic leukemia nuclear bodies (PML-NBs) into complexes that are termed APBs (for ALT associated PML bodies). Recently, we have presented results on the mobility of telomeres and PML-NBs as well as their interaction in human U2OS osteosarcoma cells, in which the ALT pathway is active. An U2OS cell line was investigated that had lac operator repeats stably integrated adjacent to the telomeres of chromosomes 6q, 11p and 12q. Via autofluorescent LacI bound to the lacO arrays the telomere/PML-NB mobility in correlation with telomere repeat length was evaluated. The results suggested that the shortening of telomeres results in an increased mobility that facilitates the formation of APBs. With this system we have further investigated the role of PML-NBs in the ALT pathway. Results will be presented that provide new insight into the assembly process of PML-NBs and APBs, their structure and composition and the mechanism of alternative telomere lengthening.

The human core and inner NAC kinetochore proteins: dynamics and architecture of their complex

Stephan Diekmann

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DNA segregation of mammalian cells is an essential cellular process mediated by a specific sub-chromosomal protein complex, the kinetochore. Malfunction of this complex results in aneuploidy and can cause cancer. In time and space in the living human cell, we elucidated the dynamics, the proximity relations and possible functions of human core and inner NAC kinetochore proteins. We measured the exchange rates of core and inner centromere proteins (CENPs) by quantitative microscopy throughout the cell cycle. CENP-A and CENP-I were found to be stable centromere components that are incorporated into centromeres via a “loading-only” mechanism in G1 and S phase, respectively. CENP-C and CENP-H are immobilized at centromeres specifically during replication. In mitosis, all inner CENPs become completely immobilized. CENPs are highly mobile throughout bulk chromatin, which is consistent with a binding-diffusion behaviour as the mechanism to scan for vacant high-affinity binding sites at centromeres. Our data reveal a wide range of cell cycle – specific assembly plasticity of the centromere that provides both stability through sustained binding of some components and flexibility through dynamic exchange of other components. We also determined the association of core and inner kinetochore proteins by FRET, FLIM and BiFC. Little information is available on kinetochore complex architecture although structural phenomena seem to play an important role for kinetochore function.

Publications:

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Automated live imaging to study human cell division

Daniel Gerlich

Fluorescence time-lapse imaging has become a powerful tool to investigate complex dynamic processes such as cell division. Automated microscopes generate time-resolved imaging data at high throughput and spatio-temporal resolution, yet appropriate analysis tools are largely missing. We therefore developed a machine learning method that combines state-of-the-art classification with temporal hidden Markov modeling for automated annotation of the progression through morphologically distinct biological states. The incorporation of time information into the annotation scheme reduced the error rate by more than ten-fold below that of single time point-based classification. Our tools enable hierarchical phenotype browsing at a screening level, as well as detailed exploration of individual movies and cells. We demonstrate generic applicability in a set of different assays and perturbation conditions, including an RNAi screen for mitotic exit timing. In conclusion, our computational tools enable live imaging-based screens and systems biology applications with ultra high-content assays.

In vivo assembly and functioning of a chromatin-associated protein complex: the chroreography of DNA repair proteins as a paradigm

Roel van Driel

University of Amsterdam and Netherlands Inst. for Systems Biology

To understand how chromatin-associated multi-protein complexes assemble and function, we have combined analysis of the in vivo kinetics of the mammalian nucleotide excision DNA repair machinery with computational modeling. We found that the components of the DNA repair machinery exchange rapidly between DNA repair sites and the nucleoplasm, whereas their net accumulation at repair sites evolved on a much slower timescale. A predictive kinetic model, constrained by these data, shows how the assembly of multiprotein complexes is orchestrated by progressive stepwise enzymatic modifications of the chromatin substrate, leaving considerable freedom for the binding mode of individual proteins. We demonstrate that the faithful recognition of DNA lesions is time consuming, while repair complexes form relatively fast through random and reversible assembly. Our system analysis reveals a fundamental conflict between specificity and efficiency of chromatin-associated protein machineries and shows how a tradeoff is negotiated through reversibility of protein binding.

ELECTRON SPECTROSCOPIC IMAGING OF CHROMATIN REVEALS SIGNIFICANT REORGANIZATION OF THE GENOME ASSOCIATED WITH INDUCED PLURIPOTENCY

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Our understanding of pluripotency has been radically affected by the remarkable discovery that differentiated adult cells can be transformed into cells like embryonic stem cells, known as induced pluripotent stem cells (iPSCs). The process of transformation, however, is currently inefficient and significant heterogeneity exists between isolated clones. This results in at least two classes of iPSCs, partial and fully reprogrammed. To date a detailed study assessing the differences between partial and fully reprogrammed cells has not been undertaken. Therefore, we set out to characterize the epigenetic factors that contribute to the variations observed among isolated iPSC clones. We obtained high-resolution structures of chromatin using electron spectroscopic imaging techniques, which unlike conventional EM, does not require the use of resolution-limiting contrast enhancement agents. Through phosphorus and nitrogen maps we are able to image chromatin fibre relationships, structural domains of heterochromatin and the intervening RNA and protein-based structures. We demonstrate that successful cellular reprogramming accompanies extensive changes to the genome itself, resulting in a global chromatin architecture that is indistinguishable from that of embryonic stem cells. Surprisingly, within the pluripotent cells, chromatin domains that are rich in heterochromatin marks, such as H3K9me3 and H4K20me3, have a degree of compaction that is indistinct from domains that do not contain these heterochromatin marks. We have further demonstrated that partially reprogrammed cells that express the Yamanaka pluripotency factors from their viral transgenes have not reorganized constitutive heterochromatin; it still has the structural features of that seen in the parental fibroblasts rather than that of embryonic stem cells. This failure to disrupt silenced genomic regions is a hallmark of their partially reprogrammed status.

We expect to generate further insights into the importance of chromatin architecture in differentiation by combining EM tomography with electron spectroscopic imaging. In so doing, the precise fibre composition and fibre-fibre interactions of compact chromatin domains can be studied.

Multiscale analysis of nanoparticle dynamics and interactions in living human cells

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We currently observe research and development of nanotechnologies on the fast lane. The number of engineered nanoparticles (NPs) and their applications grow at an equal fast pace. NPs are generated from fundamentally different materials such as amorphous silica, cadmium selenide, carbon or polystyrene, just to name a few. A wide range of chemo-synthetic methods results in an even greater variety of NP-properties. Thus, NPs exhibit characteristic qualities including particle size, morphology, composition, surface area, surface chemistry and reactivity in solution. In contrast to the growing literature on application of NPs and nanotechnology, there is little information about cellular uptake pathways and biological effects of NPs. We have recently reported that penetration of SiO_2-nanoparticles (silica-NPs) to the cell nucleus causes inhibition of replication, transcription, and cell proliferation.

In order to define the particle properties underlying these nano-bio interactions, we have here assessed the dynamics of NP distribution in vitro and in living cells using high-resolution microscopy methods including TEM, FRAP, FCS, and RICS. Applying TEM and FCS in vitro we show that NPs have a low tendency to aggregate both in water, and protein-containing solvents. We also demonstrate rapid cellular uptake of fluorescently labeled NPs within seconds. In the cytoplasm NPs are freely diffusive and they accumulate at vesicles and mitochondria. At these structures NPs turn over rapidly indicating transient binding and unbinding at membranes. In the nucleus, NPs do not aggregate as determined by FCS or RICS. However, after several hours, silica-NPs induce protein aggregation in the nucleoplasm and activation of the nuclear ubiquitin-proteasome system.

Our data provide strong evidence that nanoparticle toxicity in human cells may originate from their uptake kinetics, interaction with mitochondria and/or induction of nucleoplasmic protein aggregation. Unequivocal characterization of NP-properties by physicochemical analysis may aid in both, further elucidation of nano-bio interactions, and development of preventive strategies.

Intranuclear trafficking of native mRNA

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Messenger RNP particles (mRNPs) move randomly within the nucleoplasm before they exit from the nucleus via the nuclear pore complexes. Mobility studies of mRNPs in vertebrate cells indicated a surprisingly low particle mobility. To further understand mRNP trafficking, we have studied the intranuclear movement of a specific, native mRNP, the Balbiani Ring (BR) 2 mRNP, in salivary gland cells of *Chironomus tentans*. Their polytene nuclei harbor giant chromosomes separated by vast regions of nucleoplasm, which allowed us to study mRNP mobility without interference of chromatin by single particle tracking and fluorescence correlation spectroscopy. The particles could be fluorescently labeled with microinjected oligonucleotides (DNA or RNA) complementary to BR2 mRNA, or with the RNA-binding protein hrp36, the *C. tentans* homologue of hnRNP A1. Using high-speed, light sheet-based video microscopy we followed the intranuclear trajectories of single mRNPs, and characterized their motion within the nucleoplasm [1]. The BR mRNPs moved randomly, but unexpectedly in a discontinuous manner [2]. When mobile, they diffused with a diffusion coefficient almost corresponding to their size. Between mobile phases the mRNPs were slowed down 10 to 100-fold but were never completely immobile. Earlier electron microscopy work has indicated that BR particles can attach to thin non-chromatin fibers, which are sometimes connected to discrete fibrogranular clusters. We propose that the observed discontinuous movement reflects transient BR mRNP interactions and speculate that during these interactions (i) steps of mRNP processing or (ii) quality control before export take place, or that they serve to (iii) regulate mRNP export in response to external signals. Finally, since hrp36 is comprised in the mRNPs from the BR transcription site to the cytosolic ribosomes their route of transport including the NPC passage could directly be monitored in living cells at high time resolution.

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Translational diffusion and interaction of a photoreceptor and its cognate transducer observed in giant unilamellar vesicles by using dual-focus FCS

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The cell membrane is not only physically separating intracellular organelles and the extracellular environment but also facilitate diverse cellular functions. For example specific proteins embedded in the cell membrane can act as receptors that allow cells to communicate with each other. To fulfil these functions often larger oligomeric protein complexes need to be formed in the cell membrane. Instead of studying theses processes directly in the cell, which is often difficult or even impossible, we studied protein complex formation in large or giant unilamellar lipid vesicles which mimic a cell membrane to a certain extent. As constituent parts of a photo signalling complex the photophobic receptor from *Natronomonas pharaonis* (NpSRII) and its cognate transducer ($NpHtrII_{157}$; truncated at residue 157) was chosen and the complex formation in lipid bilayers was analyzed [1]. We reconstituted membrane proteins into giant unilamellar vesicles (GUVs) and perform spectroscopic studies using a dual-focus fluorescence correlation spectroscopy (FCS) set-up. This technique enabled us to study the formation of signalling complexes with proteins highly diluted in the lipid-bilayers (molar receptor/lipid ratio of 1:1,000,000), comparable to environmental conditions as given in cells [2].

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Molecular views on cellular oxygen sensing

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The activation of hypoxia-inducible factor-1 (HIF-1) has been recognized as the key event in adaptation to hypoxia. HIF-1 is composed of the O_2-labile α- and the constitutive β-subunit. From here the whole cascade of cellular O_2 sensing was followed back ultimately leading to the elucidation of cellular O_2 sensors, the prolyl and asparaginyl hydroxylases that regulate abundance and activity of oxygen-regulated α-subunit of HIF-1. Assembly of the HIF-1 complex then requires dimerization with constitutive nuclear β-subunit. To localize HIF-1 subunits within the nucleus of hypoxic cells we applied 2-photon-laser microscopy (2PLM). Mobility studies of fluorescently labelled HIF-1 subunits by fluorescence recovery after photo bleaching (FRAP) revealed that HIF-1α migrates more slowly than HIF-1β within the nucleus indicating that both subunits do not immediately "find" each other but may be prone to modification prior to dimerization. HIF-1 assembly was then studied in living cells in a specialized hypoxic chamber mounted on the microscopic stage which allows the in vivo analysis under a well defined oxygen tension. Assembly of the HIF-1 complex was analysed by fluorescence resonance energy transfer (FRET) and determined the distance of α- and β-subunits of the transcriptionally active HIF-1 complex bound to DNA. This organization of the HIF complex is different from so far described two dimensional models but is in line with the very recent structural consideration that heterodimer formation of the HIF complex relies on anti-parallel association of the PAS B domains within the α- and β-subunit.

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Advances In Super-resolution Imaging Technologies

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Superresolution techniques such as photoactivated localization microscopy (PALM) enable the imaging of fluorescent protein chimeras to reveal the organization of genetically-expressed proteins on the nanoscale with a density of molecules high enough to provide structural context. Various applications of this new technology are now possible. One application is for *in cellula* pulse-chase analysis to follow protein turnover and diffusion of photoactivated fluorescent proteins. Another approach combines the techniques of PALM and single particle tracking to resolve the dynamics of individual molecules by tracking them in live cells. Called single particle tracking PALM (sptPALM), the technique involves activating, localizing and bleaching many subsets of photoactivatated fluorescent protein chimeras in live cells. Spatially-resolved maps of single molecule motions can be obtained by imaging membrane proteins with this technique, providing several orders of magnitude more trajectories per cell than by traditional single particle tracking. By probing distinct subsets of molecules, including Gag and VSVG, sptPALM can provide a powerful means for exploring the origin of spatial and temporal heterogeneities in membranes. Examples such as these will be presented to illustrate the value of super-resolution imaging in providing quantitative insights into protein organization and dynamics at the nanoscale.

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TIRF microscopy evanescent field calibration using tilted fluorescent microtubules

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Total internal reflection fluorescence microscopy has become a powerful tool to study the dynamics of sub-cellular structures and single molecules near substrate surfaces. However, the penetration depth of the evanescent field, that is, the distance at which the excitation intensity has exponentially decayed to 1/e, is often left undetermined. This presents a limit on the spatial information about the imaged structures. Here, we present a novel method to quantitatively characterize the illumination in total internal reflection fluorescence microscopy using tilted, fluorescently labelled, microtubules. We find that the evanescent field is well described by a single exponential function, with a penetration depth close to theoretically predicted values. The use of in vitro reconstituted microtubules as nanoscale probes results in a minimal perturbation of the evanescent field; excitation light scattering is eliminated and the refractive index of the sample environment is unchanged. The presented method has the potential to provide a generic tool for in situ calibration of the evanescent field.

Multicolor Super-resolution SW-TIRF Microscope

Gerrit Best

Over the last years several methods were developed to evade the classic theoretical resolution limit.
Besides several Localization microscopy and confocal scanning methods the widefield method of **Structured Illumination Microscopy** is known to provide a considerable resolution improvement with a short acquisition time.
Total Internal Reflection Microscopy makes use of the tunnel effect illuminating a thin layer in the object when the excitation light is totally reflected in the coverslip.
The microscope described in the poster makes use of a unique setup to provide Structured Illumination and TIRF microscopy capability separately and in combination while using up to four different excitation wavelengths.
With these methods a resolution of down to 120nm in all spatial directions can be achieved.

How to turn an old confocal microscope into a super resolution nanoscope

Alexander Brunner, Alexander Urich, Roman Amberger, Christoph Cremer

Within a diploma thesis a confocal microscope was reconstructed into a super resolution nanoscope. Thereby the conventional confocal modus was replaced by a structured illumination modus and a localization modus. The factor of performance improvement was in the first case approximately 1.5 and in the second 6.25 in comparison to the classic wide field microscopy.

Measurement of replication structures at the nanometer scale using advanced optical microscopy

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Precise and complete duplication of the genome is essential for normal cell proliferation. Experimental determination of quantitative parameters of replicating chromatin is required for understanding and modeling of genome replication. Different ways of visualizing replication in mammalian cells yielded different estimates of DNA replication structures. Using super-resolution light microscopy techniques we have directly measured and compared the size and numbers of differently labeled DNA replication sites throughout the S-phase at the nanometer scale. The comprehensive analysis of replication foci in mammalian cells showed a significant variation of replication foci sizes (40-210 nm). However, the average size (125 nm) was conserved throughout S-phase and did not depend on the labeling method. Moreover, the improved optical 3D resolution yielded three to five fold higher replication foci numbers compared to previously reported estimates. Altogether, optical nanoscopy techniques allowed accurate measurement of genome replication structures at a level previously achieved only using electron microscopy and with the added advantage of high throughput, multispectral and 3D preservation.

Uptake of nanoparticles into epithelial and neuronal cells

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Despite of their exponentially growing use, little is known about cell biological effects of nanoparticles (NPs). A prerequisite for specific applications and assessment of potential negative health effects is to understand NP-interactions with cells. Here, we use commercially available fluorescently labeled polystyrene NPs with negative surface charges to follow the time-course of uptake and distribution of nanoparticles in different cell types by confocal laser scanning microscopy under absolute physiological conditions. Our results demonstrate that polystyrene NPs enter different types of cells and are distinctly distributed in the cytoplasm. Uptake kinetics show that particle internalization starts during the first minutes of incubation which is faster than caveolae or clathrin-mediated uptake. Treatment with nocodazole does not interfere with cellular uptake processes whereas low temperature does cause a reversible reduction of uptake. Double staining experiments reveal the colocalization of polystyrene nanoparticles with mitochondria but not with lysosomes, endoplasmatic reticulum, Golgi apparatus or microtubules, at early stages of cellular uptake processes (< 1 hour). Our results suggest cellular internalization of polystyrene NPs that is independent of caveolae- or clathrin-mediated endocytosis.

Far-field super resolution imaging of cellular components

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Far-field super resolution imaging is a fast developing microscopy branch that has the potential of replacing common fluorescence microscopy and even confocal fluorescence microscopy. We have implemented a PALM/STORM-like imaging solution on an existing fluorescence widefield microscope setup using laser light of 405, 488 and 561nm. We tested two well-characterized, established fluorescent proteins from the GFP-family for superresolution imaging, the photoswitchable Dronpa and the photoactivatable Dendra2. Dronpa was fused to the LCK membrane anchor and Dendra2 to zyxin or beta-integrin to obtain superresolution images of the cell membrane and focal adhesions, respectively. In a second attempt, we tested Alexa647 as an example of commonly used fluorescence dyes that can be driven into a dark state and switched back to the fluorescent state either by photons or by chemical means. Alexa647 coupled to Phallaodin and a secondary antibody was used for superresolution imaging of actin and microtubuli, respectively. The technical implementation and first superresolution images will be presented.

In vivo analysis of the mitotic checkpoint complex (MCC)

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During cell division, the mitotic spindle segregates the sister chromatides into two daughter cells. Each daughter cell obtains one complete set of chromosomes. Failure of this process may lead to genome instability resulting in premature aging, senescence and cancer [1,2,3].
The spindle assembly checkpoint (SAC) is a surveillance mechanism that delays anaphase onset until all chromosomes are correctly attached in a bipolar fashion to the mitotic spindle [4]. Until proper attachment of the microtubules to the outer kinetochores, the MCC (mitotic checkpoint complex) formed by BubR1, Mad2, CDC20, Bub3 is active [5]. It inactivates the anaphase promoting complex/cyclosome (APC/C) by binding to the APC/C and/or by depleting the free CDC20. Our data obtained by theoretical analysis indicate that the MCC blocks the APC/C by binding [6]. We are characterizing 30 proteins of both, the inner and the outer kinetochore. The onset of the spindle checkpoint and its regulatory interaction with the APC/C are also investigated by theoretical attempts [6,7].

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Dynamics of the active zone cytomatrix protein Bassoon: Slow turnover and regulation by Neuroligins are indicative of key roles in active zone stabilization and maturation

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The presynaptic cytomatrix is a supramolecular complex exclusively localized to neurotransmitter release sites. It is thought to act as a dynamic scaffold that enables neurotransmitter release locally, and that changes its features during development and in reponse to neuronal activity. Given the dynamic nature of many presynaptic cytomatrix components one might expect that it also includes some “anchor” molecules that are relatively stable and thus guarantee some stability in face of the developmental and activity-dependent changes occurring in the cytomatrix of individual synaptic sites.
Here, we studied the molecular dynamics of the presynaptic cytomatrix molecule Bassoon in cultured hippocampal neurons. Fluorescence recovery after photobleaching and photoactivation experiments revealed that exchange rates of Bassoon at individual presynaptic sites are very low (tau > 8 h) compared to exchange rates of other presynaptic proteins. In addition, exchange rates were only slightly increased by acute stimulation. However, the presynaptic cytomatrix underwent a developmental change in stability: at immature synapses, the presynaptic cytomatrix required F-actin to be maintained at synapses. Conversely, at mature synapses, the cytomatrix remained structurally intact in the absence of F-actin. This increase in inherent stability was accompanied by functional maturation, as revealed by live-imaging for exocytosis and endocytosis. Intriguingly, the developmental transition from immature to mature features was controlled by the postsynaptic transmembrane molecule Neuroligin 1, which has been implicated in synaptic cell adhesion and cognitive function.
Thus, our data support the notion that Bassoon constitutes a relatively stable component of the cytomatrix which may explain the tenacity exhibited by individual presynaptic sites, and that transsynaptic signaling via Neuroligins induces changes in the cytomatrix that reflect functional maturation during development.

Self-organizing actin wave patterns mimic the structure of phagocytic cups

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Actin waves generated on the substrate-attached surface of *Dictyostelium* cells are characterized by their ability to change direction. They separate two states of actin organization from each other: an inner area of dense filament assemblies dominated by the Arp2/3 complex, and an external area of loose networks of actin bundles marked by bipolar myosin-II filaments and by the anti-parallel bundling protein cortexillin. The inner area correlates with a PIP3-rich region of the plasma membrane, in support of a positive feedback cycle connecting phosphoinositides and actin polymerization.

Actin waves are generated independently of external signals transmitted through heterotrimeric G-proteins, and their propagation does not require SCAR/WAVE, a common link of signal transduction pathways to the Arp2/3 complex. The actin/PIP3-pattern of the planar waves parallels the 3-dimensional organization of phagocytic cups. Accordingly, wave-forming cells have a high capacity of taking up particles. We suggest that the waves are scanning machines in search for environmental structures of high curvature.

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High-Frequency Imaging of MyoB Dynamics in D. Discoideum

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Dictyostelium discoideum Myosin IB (MyoB) is a single-headed class I myosin that has been implicated in the regulation of pseudopod formation in general and in the suppression of lateral pseudopod formation in particular [1-4]. MyoB has been shown to associate with the plasma membrane during locomotion [5] and has been localized to actin-rich regions, as well as to the filopods of Dictyostelium cells. However, no data on how single MyoB molecules might be organized into molecular complexes and recruited to its place of action have been reported.
Using Total Internal Reflection Fluorescence (TIRF) microscopy with highly sensitive EMCCD detection, we imaged GFP-MyoB mutants in both AX2 wild-type cells and in cells having a MyoB null background. To visualize the dynamics of the single headed motor we acquired time-lapse movies using high frame rates.
Data analysis using kymographs revealed MyoB clusters along filopods as well as at the substrate attached surface of the cell body. In both cases we found the MyoB clusters moving randomly, showing no particular direction. Especially in the area of the cell body, MyoB molecules seem to diffuse as aggregates in and out of the region near the substrate attached membrane.

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Analysis of the interaction between the hypoxia-inducible factor 1α and the prolyl hydroxylase 1 by means of fluorescence resonance energy transfer

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Hypoxia-inducible factor 1α (HIF-1α) is the oxygen-responsive subunit of the transcription factor HIF-1[1] which regulates transcription of genes involved in adaptation to hypoxia [2]. In normoxia, HIF-1α is hydroxylated by prolyl hydroxylases (PHDs) 1-3 which initiates its proteasomal degradation (half-life of HIF-1α < 5 min) [3]. In hypoxia [4] or after treatment with DMOG [5], MG132 [6] and Co^{2+} [7], HIF-1α is stabilized and dimerizes with HIF-1β to form HIF-1. DMOG does not allow hydroxylation by PHDs and MG132 inhibits the proteasome. The mechanism by which Co^{2+} rescues HIF-1α from degradation is not understood until now. Since the interaction between HIF-1α and PHD1 has never been studied in living human cells, fluorescence resonance energy transfer (FRET) measurements were performed. For this purpose, cells were cotransfected with ECFP-HIF-1α and EYFP-PHD1 plasmids and exposed to normoxia. Cells were also treated with DMOG, MG132, $CoCl_2$ or DMOG and $CoCl_2$. We can for the first time directly show that HIF-1α interacts with PHD1. Interestingly, this interaction was only observed after addition of DMOG. When no reagents or MG132 are added, the interaction between overexpressed HIF-1α and PHD1 is presumably too short-lived although degradation of HIF-1α is slowed due to overload of the proteasome. The fraction of HIF-1α and PHD1 proteins interacting during microscopy is still too low. DMOG obviously enhances this fraction which indicates a longer and/or repeated interaction. $CoCl_2$ negatively influenced the DMOG effect not due to quenching effects but due to inhibiting the interaction between HIF-1α and PHD1.

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Dual Color Localization Microscopy of Cellular Nanostructures

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Fluorescence microscopy methods have played a major role in the elucidation of the structure of the cell nucleus. However, a major limitation was the optical resolution thought to be restricted to a fundamental limit of ca. 200 nm (Abbe/Rayleigh limit). Consequently, the chromatin nanostructure was not accessible to light microscopy.
Various recently introduced laseroptical “nanoscopy” approaches such as Structured Illumination (SI), 4Pi- and Stimulated Emission Depletion (STED) Microscopy allowed to extend the spatial analysis far beyond these limits. Here, the focus will be on a complementary method, “Spectrally Assigned Localization Microscopy” (SALM). SALM is based on labelling ‘point like’ objects (e.g. single molecules) with different spectral signatures, spectrally selective registration and high precision localization monitoring by far field fluorescence microscopy. A recently developed SALM procedure, Spectral Position Determination Microscopy (SPDM) permits the spatial analysis of large numbers of molecules at high intracellular densities in combination with widely used fluorescent proteins and synthetic dyes. Presently, the SPDM method allows us ‘nanoimaging’ of two molecule types in the same cell (“Dual Color Localization Microscopy”, 2CLM).
The capabilities of 2CLM for studying the spatial organization of the genome in the mammalian cell nucleus are demonstrated for the relative distributions of the DNA remoddeler protein Snf2H labeled with standard GFP and the histone protein H2A labeled with mRFP1 in human U2OS osteosarcoma cells. The 2CLM technique revealed quantitative information on their distribution in the cell nucleus as well as their spatial relationships such as their colocalization and anti-colocalization down to length-scales of 30 nm.

Quantitative imaging of core kinetochore and NAC protein assembly in living human cells

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The centromere/kinetochore complex specifies a DNA/protein assembly at the surface of chromosomes essential for faithful DNA segregation. Many of the proteins involved in kinetochore formation are known, however, little information is available on the dynamics of this complex chromosomal substructure although its architecture seems to play an important role for kinetochore function. We apply fluorescence bleaching (FRAP), correlation (FCS, RICS), resonance energy trasnfer (FRET), lifetime imaging (FLIM) microscopy on FP-tagged centromere proteins in living human cells to unravel the interaction network, architecture and assembly dynamics of the human centromere in time (cell cycle) and space. We determined the association network (in the < 10 nm range) of inner kinetochore proteins as well as histones in living human HEp-2 cells by FRET, BiFC and FLIM.
Using FRAP and FCS we identified CENP-A and CENP-I as hyperstable centromere components which are incorporated into centromeres via a 'loading only mechanism' in G1 and S phase, respectively. All other components showed protein-specific exchange rates at centromeres and a binding-diffusion behaviour as the mechanism to scan for vacant high-affinity binding sites at centromeres. Thus, centromere architecture is based on components which provide both, stability through sustained binding of some and flexibility through dynamic exchange of other components. We continue this approach on all other kinetochore proteins in order to obtain the full range of assembly dynamics of the human centromere/kinetochore complex. This will help us to elucidate the mechanisms responsible to build this dedicated structure on human chromosomes.

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The kinetochor protein CENP-W: localization, dynamics and neighbourhood relations

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Faithful chromatin segregation is mediated and controlled by the kinetochore network, which assembles at the centromere. Centromeric chromatin consists of specialized interspersed regions, in which histone H3 is replaced by the H3 variant CENP-A (CenH3). The structural core of the kinetochore contains the proteins of the CCAN (constitutive centromeric associated network), among them are CENP-C, CENP-T, CENP-M, CENP-N, CENP-U. CENP-H, CENP-C, CENP-I, CENP-Q, CENP-R, and CENP-S. We could show by acceptor-bleaching FRET (Förster Resonance Energy Transfer) that CENP-U is located close to CENP-I and CENP-B, and that CENP-T is in proximity to CENP-M.[1)] Recently the new CCAN protein, CENP-W, was discovered.[2)] CENP-W forms a complex with CENP-T. This complex associates with centromeric DNA and with histone H3, but not directly with CENP-A.

In this study we analyzed the localization, the dynamics, and the neighbourhood relations of CENP-W in living human cells. CENP-W is constitutively (during the complete cell cycle) located at the kinetochore. By FRAP (Fluorescence Recovery After Photobleaching) analysis we could show that CENP-W is stably bound and hardly mobile. FRET analysis revealed a close vicinity of the N-terminus of CENP-W to CENP-T in living cells. We analyze further the neighbourhood relations of CENP-W and CENP-T to histone H3 variants H3.1, H3.2, and H3.3.

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Use of ultrahigh resolution fluorescence microscopy to analyze the localization pattern of p-glycoprotein

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A major problem to treat cancer, numerous central nervous system diseases, and AIDS, is to approach drugs inside the brain. The transport of xenobiotics from blood to brain is represented by the brain capillaries, which build the blood brain barrier (BBB). Many drugs are not able to penetrate the BBB because they are transferred back to the blood by ATP binding cassette (ABC) export proteins being identified in brain capillaries. Overexpression of the ABC transporter p-glycoprotein (pgp), the *MDR1* gene product, confers multidrug resistance (MDR), which is one of the major causes for chemotherapeutic treatment failure in cancer patients. Therefore, manipulation of pgp is a necessary approach to circumvent MDR. Little is known about the organized localization of pgp within the luminal membrane of the BBB.
In the current work, we explore the expression pattern of EGFP (Enhanced Green Fluorescence Protein)-pgp fusion protein transfected in an immortalized cell line of Rat Brain Endothelial (RBE4) cells. This is achieved by means of the fluorescence microscopy method Spectral Precision Distance Microscopy/Spectral Position Determination Microscopy (SPDM) with single fluorescent molecule resolution developed in the Cremer laboratory in the Kirchhoff Institute for Physics, University of Heidelberg. While electron microscopy offers an extremely high resolution, light microscopy shows great advantages, like identification of different types of labeled molecules in intact, three-dimensional cells.
SPDM has the potential to circumvent the optical resolution limit. In order to obtain structural information far below the optical resolution limit, the single fluorescent molecules of the structure have to be detected independently. This can be realized by optical isolation, which means that in a given optical resolution-limited observation volume at a given time, only one such object (*i.e.* EGFP-pgp fusion protein) is registered[1].
Here we report that the transfected RBE4 cells can be identified with a spatial localization accuracy of 35nm in average. This offers a resolution improvement of more than 80% compared to conventional far-field microscopy. Furthermore, the expression pattern of the EGFP-pgp chimera in RBE4 cell plasmamembrane can be determined. These fusion proteins are arranged in clusters of 30nm size. However, single cluster organization shows a random distribution. Further studies should confirm the here shown results under normal as well as under pathological conditions.

Fast biosynthesis of GFP molecules – a single molecule fluorescence study

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Numerous studies showed that protein folding and maturation can differ substantially between *de novo* synthesized proteins and *in vitro* refolded proteins. In classical folding studies folded proteins are initially denatured into an unfolded state before the (re-) folding process can be studied. It has been demonstrated that protein folding takes place already during the elongation of the nascent chain (co-translational folding).

Here we present an approach employing a two color single molecule sensitive fluorescence wide-field microscope in order to visualize surface tethered fluorescently labeled ribosomes and *de novo* synthesized GFP molecules in real time[1]. Suppression of protein release after synthesis keeps the synthesized GFP bound to the ribosome and allows to image GFP fluorescence for extended observation times.

We demonstrate that the green fluorescence protein mutant GFP Emerald (GFPem) is produced with a characteristic time of five minutes, which is one of the fastest maturation times for a GFP mutant observed so far. The fastest GFP molecules appear already within one minute. Processes precedent to chromophore formation, such as polypeptide synthesis and protein folding, are fast and last less than one minute. High rates of folding and maturation are assumed to improve the efficiency of protein synthesis in the cell.

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Simulations on Localization Microscopy for differently labelled Regions

Rainer Kaufmann

SPDM (spectral precision distance / position determination microscopy) is a method of localization microscopy by using conventional fluorophores. Based on a reversibly bleached state of the molecules, this method not only allows use to resolve structures in the 20 nm regime far below the resolution limit (Abbe/Rayleigh), also single molecule information is provided. For every detected molecule the position within the localization error is known. This information, which is not given in other super-resolution light microscopy techniques, can be used for statistical analyses of the molecule distribution. An example therefore is two differently labelled regions, whose distance (or overlap) can be precisely determined via localization microscopy. Simulations show, what is possible and what are the requirements for certain conditions.

Isoform-specific chromatin binding of heterochromatin protein-1 in living cells

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Heterochromatin maintenance is crucial for proper chromosome segregation, clonal inheritance of cell identity and the regulation of gene expression. Although architecturally stable, heterochromatin has to be flexible to cope with biochemical events on DNA. One possible mechanism underlying functional flexibiliy of heterochromatin relies on short residence times of heterochromatin protein 1 (HP1) isoforms [1-3]. To further dissect individual contributions of HP1 isoforms to heterochromatin maintenance we have assessed the dynamics of HP1α, HP1β, and HP1γ by live-cell microscopy and mathematic modeling.

Our results indicate that a considerable fraction of all three HP1 isoforms diffuses freely within the nucleoplasm. The chromatin-binding population is divided into two major classes, a fast one with residence times of ca. 5 sec, and a slower one with residence times in the minute range. HP1γ exhibits more stable binding in euchromatin than HP1α and HP1β. In heterochromatin the residence time of the slow fraction of HP1β is 3 to 5-fold higher than for HP1α and HP1γ. The apparent diffusion coefficients of all HP1 isoforms as assessed by FCS and RICS in euchromatin (D = 1.3 $\mu m^2 s^{-1}$) and heterochromatin (D = 0.6 $\mu m^2 s^{-1}$) are almost identical. We also show that loss of the chromo domain or the chromoshadow domain renders HP1b unable to form the slow exchanging population, indicating that the domains act in concert for proper chromatin binding. The hinge region does not contribute to chromatin binding at all.

Our observations for the first time indicate isoform-specific chromatin dynamics of HP1 proteins in living cells probably reflecting some individual duties on chromatin.

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PROTEIN DISTRIBUTION AND DYNAMICS IN NUCLEAR COMPARTMENTS

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Genome activity and nuclear metabolism clearly depend on accessibility, but it is not known whether and to what extent nuclear structures limit the mobility and access of individual molecules. We used fluorescently labeled streptavidin (coupled to a nuclear localization signal by biotin) and ovalbumin as average-sized, inert proteins to probe the nuclear environment. The proteins were injected into the cytoplasm of mouse cells, and single molecules were tracked in the nucleus with high-speed fluorescence microscopy. We analyzed and compared the mobility of single streptavidin and ovalbumin molecules labeled by Cy5 respectively by Atto647N in structurally and functionally distinct nuclear compartments of living cells. Our results indicated that all nuclear subcompartments were easily and similarly accessible for such average-sized proteins, and even condensed heterochromatin neither excluded single molecules nor impeded their passage. The only significant difference was a higher frequency of transient trappings in heterochromatin, which lasted only tens of milliseconds. The probe molecules, however, did not accumulate in heterochromatin, suggesting comparatively less free volume. Interestingly, the nucleolus seemed to exclude both streptavidin and ovalbumin, as it did many other nuclear proteins, when visualized by conventional fluorescence microscopy. The tracking of single molecules, nonetheless, showed no evidence for repulsion at the border but relatively unimpeded passage through the nucleolus. These results clearly show that single-molecule tracking can provide novel insights into mobility of proteins in the nucleus that cannot be obtained by conventional fluorescence microscopy [1, 2]. Our results suggest that nuclear processes may not be regulated at the level of physical accessibility but rather by local concentration of reactants and availability of binding sites.

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Nuclear import and export in living cells examined one molecule at a time

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All molecular traffic between cytoplasm and nucleus occurs via nuclear pore complexes (NPCs), which are huge supramolecular transporters spanning the nuclear envelope of eukaryotic cells. Using single molecule observation based on classical epi-illumination fluorescence microscopy we were able to determine the dwell times of several different transport receptors in digitonin-permeabilized cells [1] and also in live cells [2]. The great majority of all receptors resided less than 20 ms at the NPCs, but, surprisingly, we found also that a small fraction of all examined transport receptors has conspicuously long residence times at the NPC.

Recently, we focused on a detailed characterization of the transport kinetics of nucleocytoplasmic transport of probes ranging from import and export receptors (NTF2, transportin, CRM1 and CAS) to cargoes such as mRNA (BR2 mRNP) and transcription factors (STAT1). In order to optimize the signal-to-noise ratio while simultaneously reduce photobleaching of the precious single molecule fluorescence we used advanced microscopic techniques such as HILO microscopy [3] and SPIM [4] at millisecond time resolution. In a modified HILO experiment we illuminated the bottom of cell nuclei with a refracted beam. This refracted beam was created by hitting the coverslip/sample interface at an angle just slightly smaller than the critical angle of total reflection. Thereby only the NPCs at the very bottom of the nuclei close to the objective lens were illuminated thus strongly reducing the background produced by out-of-focus fluorescence. This greatly simplified the identification of single NPCs and strongly reduced the irradiance of single fluorescently labeled transport receptor molecules prolonging their overall observation time window. A similar effect was obtained using light sheet based microscopy [4]. We illuminated the sample perpendicular to the detection axis with a thin light sheet of an axial thickness of 2-3 µm only. In this manner a simple optical sectioning microscope was created, because only the focal plane of the detection optics was illuminated and no out-of-focus fluorescence was generated, what greatly improved the signal-to-noise-ratio. In order to improve the applicability of the method, we constructed a miniaturized glass specimen chamber, which could be illuminated from the side in a very flexible manner and directly be mounted on a commercial inverse microscope. The specimen was easily accessible from above for micromanipulation and could be observed via the 0.17 mm thin glass bottom of the chamber using high NA objective lenses. Using this setup, we observed and analyzed the nucleocytoplasmic transport of single transport receceptors and mRNA molecules with unusually high contrast in living Cironomus tentans salivary gland cell nuclei.

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Short Actin Filaments for Fast Movement

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Plasmodium sporozoites are the forms of the malaria parasite transmitted from mosquitoes to a vertebrate host. Even after years of investigation one essential process required for pathogenesis, the motility of the parasite, remains in its molecular detail and mechanism largely uncharacterized. Actin is one crucial factor for this motility. Various drugs, such as cytochalasin D and jasplakinolide, inhibit actin dynamics and thus modulate the motility of the parasite[1]. However, the polarity of the actin filaments and their orientation within the parasite, which are both important for the understanding of the cell movement, have not been studied in detail. Investigations have been hampered due to the shortness of the actin filaments and the fact that only a minor part of the total actin pool of the cell polymerizes into filaments[2]. New imaging techniques are needed to tackle this problem. In order to get a better view on the gliding machinery we investigated whether it is possible to correlate the *in vitro* gliding behavior with cryo-electron tomography. The parasites readily glide on electron microscope (EM) grids and can be directly imaged using light microscopy. Following the grids were plunge frozen and at a later time point transferred to the EM. Our study reveals that the adherent properties of cells are crucial to allow a correlative approach. Further we were not able to detect actin filaments independent of whether the parasites were previously gliding on the grid or not.

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Structural basis of synaptic vesicle organization and release revealed by cryo-electron tomography

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The presynaptic terminal contains a complex network of filaments whose precise organization and functions are not yet understood. In this study, we combined pharmacological treatments that induce specific perturbation in the synaptic release machinery with cryo-electron tomography, a method allowing visualization of synaptic molecular complexes free of artefacts induced by chemical fixation, aggregation and non-uniform distribution of heavy-metal staining. As a result, we obtained objective detection and quantitative characterization of molecular strands linking synaptic vesicles to each other and to the active zone, and provide a structural model covering some aspects of the synaptic vesicle mobilization and neurotransmitter release.

Observation of single transport events through single pore complexes of the nuclear envelope by means of near-field optical microscopy

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We solved a major obstacle of scanning near-field optical microscopy (SNOM) imaging on soft matter in solution by establishing a novel distance control based on the weak elastic response of a freely suspended membrane patch [1]. This method enabled us to reliably image a native nuclear envelope (NE) of *Xenopus laevis* oocytes at high optical resolution. As the transport channel of the NPCs was no longer obstructed by a support, the translocation of proteins through a single pore could be studied on a microsecond time scale by placing the near-field probe over a pore. The extremely short depth of the evanescent field in axial direction of ~10-25 nm generated a steep intensity gradient along the axis of the transport channel, so that fluorescently labeled nuclear transport factor 2 (NTF2) produced strong fluorescence fluctuations while passing through the pore. These fluctuations were evaluated by means of a fluorescence correlation analysis based on a model of Brownian motion in a confined space. In that way, the features of SNOM, ultra-high spatial and temporal resolution, were employed to reveal the kinetics of a transport process on a single-molecule level.
As a primary achievement, we can report first experimental data regarding the rapid mobility of a protein within the transport channel of the NPC [1]. The good agreement between a simulation of confined diffusion of NTF2 in a channel and the experimental data strongly supports a model for a facilitated translocation of NTF2 that is based on Brownian motion as driving force. The measured diffusion coefficient at the channel entrance is comparable to the diffusion coefficient of NTF2 in proteinous solutions. Though trapped in the pore, NTF2 turned out to have a high degree of mobility within a large axial extension along the transport channel. Our findings support the idea that molecules in transit interact with FG-repeat domains at the periphery of the channel and thus favor transport models proposing such interactions. Furthermore, we could determine the residence time of NTF2 within the pore and found a good correspondence with results of previous studies based on single molecule tracking techniques.

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SPDM – Single Molecule Super-resolution of Receptor Clusters in E. coli Bacteria

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Localization microscopy based on principles of SPDM (spectral precision distance microscopy)[1] and related methods ('spectrally assigned localization microscopy', SALM) has been considerably improved by several groups, especially by using photoconvertable fluorochromes. Here we implemented SPDM in the nanometer resolution scale to a conventional fluorochrome that shows 'reversible photobleaching'. Their reversible photobleaching is used for an optical isolation in time.

Chemoreceptors and cytoplasmic chemotaxis proteins in *Escherichia coli* form clusters that play a key role in signal processing. These clusters localize at cell poles and at specific positions along the cell body which correspond to future division sites, but the details of cluster formation and the mechanism of cluster distribution remain unclear [2]. The chemotaxis phosphatase protein CheZ, labelled with YFP, was expressed under control of pTrc promoter induced with 50µM IPTG (isopropyl-beta-D-thiogalacto-pyranoside) in *flgM Escherichia coli* cells with upregulated levels of all chemotaxis proteins. The fusion protein localized to receptor clusters at the cell poles, which mediate bacterial chemotaxis. The bacteria were attached to standard cover slips pre-coated with Poly-L-Lysin (Sigma-Aldrich), fixed with paraformaldehyde following a standard protocol and embedded in Fluoromount-G (SouthernBiotech). An average localization precision of 22nm was achieved.

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Widefield-Multiparameter FLIM by a space sensitive single photon counting detector

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Understanding functional changes within living cells requires a precise investigation of the molecular interactions between macromolecules within their natural environment. The introduction of 4pi-microscopy, STED microscopy, spectral precision distance microscopy and PALM/STORM in combination with point-spread function engineering have shown that it is possible to overcome the limits of spatial resolution of far-field microscopy and to fill the gap between the molecular level (1-10 nm scale) as detected by fluorescence resonance energy transfer FRET-techniques and the confocal domain (>200 nm, laterally). Unfortunately, most fluorescence microscopes work at high excitation intensities. The strive to collect as many photons as possible during short exposure times and the application of scanning systems (often using fs-lasers), necessarily results in high average and peak-powers (typically >1 MW/cm^2), which leads to irreversible photobleaching of dyes and significant photodamage of living cells. Therefore, most of the current visualisation techniques are limited in the direct observation of molecular interactions over a longer period of time, and may induce photodynamic reactions of the molecular probes. Prerequisite for non-distorted visualisation of dynamic processes within living cells (e.g. during synapse formation and signal transduction) by fluorescence microscopy, is a minimal-invasive approach to excite and detect the molecular constituents at conditions that preserve the undisturbed living state.
In the current work we present a novel imaging system to acquire long-term multi-parameter information of living cells. The system consists of a conventional fluorescence microscope combined with a space sensitive single photon-counting detector (an improved version of a Quadrant Anode photomultiplier) and wide-field pulsed interleaved laser excitation.
In contrast to conventional detectors (EM-CCD, PMT, APD), which provide high quality intensity based images our system is able to acquire and store the full set of system parameters, completely describing the physical properties of each individual photon:

$$h\nu = f\,[x,\ y,\ \Delta t_{(TAC)},\ t_{(abs)},\ \lambda_{(em)},\ \|/\perp]$$

where x, y are the space coordinates of the impinging photon at the photocathode, $\Delta t_{(TAC)}$ the time difference correlating laser excitation pulse and fluorescence photon (yielding ps/ns fluorescence dynamics), $t_{(abs)}$ the absolute arrival time of each fluorescence photon at the detector (resulting in tracking capabilities and diffusional rates), $\lambda_{(em)}$ its emission wavelength (leading to time-resolved emission spectra), and $\|/\perp$ the direction of polarisation (providing anisotropy dynamics). In addition, it has the advantage to achieve the minimum possible excitation intensity at a given illumination energy dose, i.e compared to LSM the peak intensity is reduced by factor 1,000 to 10,000.
Applications of this widefield multiparameter fluorescence lifetime imaging microscope for long-term live cell imaging and FRET will be presented.

Where to dispose the proteins in transcription?

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Transcription is the first step in gene expression and hundreds of proteins must interact with a small region of the DNA strand to successfully transcribe one gene. It is essential for the cell to coordinate and regulate these proteins: all players must be at the right time in the right place to obtain correctly transcribed nascent RNAs. One helpful nanomachine in this complex regulation process is the proteasome. Many components of the transcription machinery are degraded by the proteasome, e.g. the RNA pol II or transcription activators [1,2], but the spatial structure-function relationship between the proteasomal proteolysis and transcription is unknown.
Nuclear proteasomes are active in distinct domains: proteins in the nucleus are degraded in proteolytic foci or centers [3]. A chromatin profiling shows that active proteasomal proteolysis is not localized in heterochromatic regions of the nucleus, but is clearly visible in the euchromatin which hosts nuclear processes such as DNA methylation, chromatin remodelling and the transcription. To resolve the spatial structure-function relationship of the active proteasomal proteolysis and transcription we use a dynamic localization-approach: (1) Active proteasomal proteolysis is analyzed in relation to global transcription in modulated cell systems and local transcription in single cells. (2) The mobility of proteolytic foci enables us to investigate the dynamic interaction of both processes. Thus, the dynamic contribution of proteasome-dependent proteolysis to global and local transcription will be discussed.

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Analysis of cell cycle dependent dynamics of Dnmt1 by FRAP and kinetic modeling

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Fluorescence recovery after photobleaching (FRAP) is an effective tool to study the mobility of molecules in living cells[1, 2]. Here we present an improved protocol for half nucleus FRAP experiments linked with a refined compartmental modeling to extract quantitative information about underlying physical binding parameters. We applied this method to analyze the cell cycle dependent kinetics of the maintenance DNA methyltransferase 1 (Dnmt1), an essential epigenetic factor that reestablishes methylation of hemimethylated CpG sites generated during DNA replication in S phase[3]. Two domains of Dnmt1, the PCNA binding domain (PBD) and the targeting sequence (TS)-domain have been shown to be responsible for targeting to replication sites and to constitutive heterochromatin[4-6]. However, their cell cycle dependent coordinated action and regulation is still unclear.
FRAP analysis and modeling of the wild type Dnmt1 fused to GFP (GFP-Dnmt1) expressed in somatic mouse cells revealed only one dynamic population in G1, whereas in early S phase an additional slower population was identified. Analyzing a PCNA binding deficient mutant (GFP-Dnmt1^{Q162E}) we found near identical kinetics in G1 and early S-phase indicating that the PBD mediated interaction is the major contribution to the decreased mobility in early S phase. In contrast, in late S phase we observed an additional decrease in the mobility of wild type and PBD mutant Dnmt1, reflecting the onset of the TS-interaction in the transition to late S phase. Consistently, a GFP-Dnmt1$^{Q162E/\Delta TS}$ double mutant shows fast recovery rates throughout S phase comparable to wild type Dnmt1 in G1.

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Studying PML nuclear body assembly by light microscopy

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The promyelocytic leukemia protein (PML) localizes to distinct nuclear bodies, referred to as PML nuclear bodies (PML NBs). PML is involved in DNA repair, apoptosis, gene regulation and senescence, but little is known about how this protein mediates these functions. As a further step to gain more insight into PML function we studied PML nuclear body formation and quantified the subnuclear distribution of PML under various stress conditions and by light microscopy.

We demonstrate that all six nuclear PML isoforms are individually able to form nuclear bodies in the absence of the other isoforms. PML NB number and morphology is highly sensitive to quantitative expression of individual PML isoforms. Only PML IV is able to induce PML microbodies. Sp100 forms nuclear bodies in the absence of PML and these structures recruit typical PML NB components suggesting that nuclear body formation does not necessarily rely on the presence of PML molecules.
Furthermore we show that PML Nbs contain only little amounts of the available PML molecules in the nucleus, more than 90% of PML is localized diffusely throughout the nuclear volume. On the other hand, PML is concentrated in nuclear bodies by a factor of 10 to 40 compared to its concentration in chromatin. These observations support a functional model in which 'soluble' PML can act within chromatin as well as exhibiting polyvalent scaffolding at nuclear bodies. The latter ensures potentiation and control of specific biochemical activities within PML NBs as a result of PML (or Sp100?) selfassembly.

Localization and dynamics of BubR1 subdomains, the central regulator of mitotic progression

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Human BubR1 is considered to play a central role in regulating several cellular processes such as mitosis related tasks, senescence, organismal aging and cancer prevention.
Stressful stimuli (DNA damage, telomere maintenance defects) lead to the onset of senescence (permanent cell cycle arrest). Animals expressing insufficient levels of BubR1 have a significantly reduced lifespan and exhibit progeroid features. BubR1 hypomorphism leads to aberrant chromosome segregation that might activate cascades establishing the senescent state and driving the aging process[1]. Premature sister-chromatid separation can also result in aneuploid cancers, highlighting the key role of BubR1 in the spindle checkpoint[2/3].
BubR1 is primarily known as (i) a component of the SAC (spindle assembly checkpoint) localizing at unattached kinetochores and (ii) as the major regulator of the MCC (mitotic checkpoint complex) inhibiting Cdc20, the co-activator of the APC/C. The APC/C regulates mitotic progression by targeting mitotic substrates for proteasomal degradation[4/5].
Human BubR1 features multiple domains that enable its functional variety[4]. It has been implicated in mitotic checkpoint control, mitotic timing and stable chromosome-spindle attachment[4/6].

Therefore we cloned BubR1 subdomains and several deletion mutants and analyzed their localization properties as well as their dynamic behaviour *in vivo* by fluorescence life time imaging (FRAP). We determine their interactions via Yeast two hybrid and their proximity to other proteins *in vivo* by FRET.

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The role of PML nuclear bodies in the regulation of MHC class II gene expression

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Promyelocytic leukemia nuclear bodies (PML NBs) are subnuclear protein assemblies involved in a variety of biological functions including apoptosis, senescence, DNA repair, and anti-viral response. The precise biochemical function in these processes is not known. PML NBs may be sites of storage, posttranslational modification or assemblies of specific nuclear protein complexes [1]. They accumulate a variety of transcription factors [2] and can spatially associate with transcriptionally highly active genomic regions [3]. These and other observations [4.5] suggested that they may directly regulate transcription of specific genes.

Here we demonstrate that interferon gamma (IFNγ) induces an association between PML NBs and the major histocompatibility class II (MHC-II) gene cluster. This genomic region encodes proteins essential for the cellular immune response (antigen processing, antigen presentation) and is essentially regulated by the MHC-II transactivator CIITA [6]. Coexpression of some MHC-II specific transcription factors (CIITA, RFXB, RFXAP, & NFYA) with PML results in their colocalization with the PML NBs. Most importantly, PML depletion leads to destabilization of CIITA and subsequently to a reduced mRNA level of some MHC-II genes, thus identifying PML as a coregulator of MHC class II expression.
This is the first demonstration of a direct link between PML, the nuclear bodies, and the MHC class II transcription machinery.

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Analysis of the three-dimensional structure of the nucleosome associated kinetochore complex

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The vertebrate kinetochore is a highly conserved complex structure, which mediates the binding of spindle microtubules to the chromosomes, thus being essential for accurate chromosome segregation. With electron microscopy, a three-laminar structure composed of an electron-dense inner plate, a less electron-dense layer and an electron-dense outer plate can be seen in mitosis.[1] The inner kinetochore is composed of several protein complexes which maintain the structural basis, whereas the dynamical outer kinetochore functions as a mechanical link to the microtubules and the components of the mitotic spindle checkpoint.[2-5] During interphase, the kinetochore is assumed to form a globular structure. Furthermore, the centromeric nucleosomes might be tetrameric instead of octameric.[6] More detailed structural information is crucial for a deeper understanding of kinetochore function.

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Dictyostelium discoideum **cytoskeletal dynamics in response to high-resolution temporal stimuli**

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It was the aim of this study to characterize intrinsic time scales of the actin cytoskeleton in chemotactic cells. Our experiments were performed with the social amoeba *Dictyostelium discoideum*, a popular model organism for the study of actin dynamics *in vivo*. Under starvation, *D. discoideum* senses extracellular cAMP by a G-protein coupled signaling cascade, which stimulates actin polymerization and re-organization of the cytoskeleton. Polymerization of actin fibers within the branched cortical network exerts a force at the membrane of the leading edge resulting in the formation of pseudopods and, finally, cell motion.
In this study, we have investigated the cytoskeletal responses of starvation developed *Dictyostelium* cells to short-time periodic stimuli of cAMP. We used laser mediated photo-activation of DMNB-caged cAMP in a microfluidic channel to generate short (1 sec) cAMP stimuli with periods between 6 s and 40 s. Responses of the actin
cytoskeleton were imaged by confocal laser scanning microscopy of a LimE-GFP *D. discoideum* construct. We performed a frequency analysis of the fluorescence signal and applied standard cell tracking tools. Frequency doubling in the cytoskeletal responses as well as chemotaxis towards the periodically active source of cAMP was observed. The data analysis was complemented by numerical finite element simulations. Furthermore, experiments were performed using single-pulse stimulation of cytoskeletal mutant strains with deficiencies in Aip1 and coronin. Our results indicate changes in the characteristic time scale of actin depolymerization. Future work will focus on periodic stimulation of these and other mutant strains.

Atomic-scale magnetometry using single defects in diamond for superresolution microscopy

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Diamonds contain natural defect centres in their lattice structure known as color centres. Electron spin states in these color centres can be changed and measured with optical techniques at room temperature.[1] The potential of locating one of these centres spatially on the nanometer scale with potential for sub-nm precision by using magnetic resonance techniques has been recently shown by our group and collaborators.[2] Using small diamond nanocrystals containing a NV-centre (nitrogen with adjacent vacancy) we intend to implement diamond as non-toxic biological marker[3] having potential to overcome the classical resolution limit of light microscopy under physiological conditions.

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