Workshop on
Cell Biology & Microscopy

... for phD students and young postdocs

Mo 02. – Fr 06. Juni 2008

Youth hostel
Burg Altleinigen,
(Grünstadt an der Weinstraße)

Organisers: S. Diekmann and C. Cremer

Goal of the workshop: presentation and discussion of own results
(every participant presents a poster)

Topics: Live cell imaging, FRAP, FCS, FRET, FLIM, single particle tracking, new microscopy techniques, CryoEM, AFM, RNAi, stable cell lines & transfection

presented in shorter talks by the participants

and 3 senior talks

arrival: monday, 02.06.2008, noon, departure Friday 06.06.2008, noon

costs: no registration fee, no participation fee; participants have to cover her/his own travel and accomodation costs, accomodation: 28 € per day (bed & meals)

application to: S. Diekmann via e-mail: diekmann@fli-leibniz.de or
Fax: +49 (0) 3641- 65 62 61 application deadline: 15.04.2008

Workshop *Cell Biology and Microscopy*

Monday 2.6.2008	Tuesday 3.6.2008	Wednesday 4.6.2008	Thursday 5.6.2008	Friday 6.6.2008
	Session 1 9:00 h R. Veith 9:40 h J. Ritter 10:20 h A. Veenendaal break 11:00 h U. Schmidt-Ziffels 11:40 h I. Chung	Session 3 9:00 h A. Scharf 9:40 h L. Singer 10:20 h I. Larson break 11:00 h A. Pluskota 11:40 h J.C. Meile	Session 6 9:00 h T. Ulbricht 9:40 h S. Münch 10:20 h K. Klement break 11:00 h D. Hellwig 11:40 h C. Weber	take down posters Departure
	12:30 h – 13:30 h Lunch	12:30 h – 13:30 h Lunch	12:30 h – 13:30 h Lunch	
Arrival	Poster Session 14:00 h – 15:30 h	Posters, free	Posters, free	
Check in				
	Session 2 15:30 h J. von Hase 16:10 h P. Lemmer	Session 4 15:30 h E. Baumgart 16:10 h P. Müller	Session 7 15:30 h A. Rump 16:10 h W. Jiang	
Put-up posters	16:50 h T. Ruckelshausen 17:30 h M. Gunkel	16:50 h A. Pinto 17:30 h A. Groß	16:50 h R. Kumar 17:30 h V. Döring	
18:30 h – 19:30 h Dinner	18:30 h – 19:30 h Dinner	18:30 h – 19:30 h Dinner	18:30 h – 19:30 h Dinner	
Opening Lecture 20:00 h C. Cremer			Closing Lecture 20:00 h M. Hausmann	

According to demand, we can have discussion rounds on selected topics (like for example: properties of fluorescent proteins, the Abbe limit, application of new microscope techniques, comparison of biochemical with microscope techniques, etc.).

List of Participants

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Intranuclear Dynamics of Single mRNA Molecules In Living *C. tentans* Salivary Gland Cell Nuclei

Roman Veith

The salivary gland cells of the dipteran *Chironomus tentans* provide an elegant model system for the analysis of specific messenger ribonucleoprotein particles, the Balbiani Ring (BR) mRNPs. BR2 mRNPs contain long RNA transcripts (~35-40 kb) and are roughly 50 nm in diameter. Although their biogenesis was thoroughly analysed, up to now very little is known about the intranuclear dynamics of the BR mRNPs *in vivo*. We analysed the intranuclear mobility of BR particles by single particle tracking of fluorescence labelled BR2 mRNPs in living gland cells. Labelling was accomplished by microinjection of fluorescent oligonucleotides, which were complementary to a highly repetitive sequence on the BR mRNA. Using high speed laser microscopy we could for the first time follow the intranuclear pathways of native mRNPs. They exhibited a very complex *in vivo* dynamics, but did not show any sign of directed motion. Fast particles moved with a diffusion coefficient of up to 4 µm²/s, slower particles with 0.6 and 0.2 µm²/s. A forth fraction was almost, but not completely immobile with 0.015 µm²/s. Using inert fluorescence nanoparticles and quantum dots the nuclear viscosity was measured as ~ 4 cP. Thus, the fast mRNPs could move as rapid as expected by hydrodynamics of non-interacting 50 nm particles. Hence, they can cover the distance from the transcription site to the nuclear envelope within the huge salivary gland cell nuclei with diameters of 60 µm within 1 minute only. Slow mRNPs were supposed either to be transiently retarded or comprised in slow, bulky supramolecular complexes. Functionalized fluorescent quantum dots were used to study the transient mRNP interactions.

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The role of the proteasomal proteolysis in transcription

Andrea Scharf and Anna von Mikecz

The nuclear ubiquitin-proteasome system (nUPS) is active in distinct nuclear domains. Proteolytic foci or centers are localized in the euchromatin that hosts nuclear processes such as the initial steps of gene expression, DNA methylation and chromatin remodelling. Despite the mounting evidence for a connection between transcription and the nUPS, the role of active proteasomal proteolysis in transcription is largely unclear. We imaged transcription in single cells using an inducible single locus-specific reporter system that allows us a precise colocalisation study with proteasomes and proteolytic foci. The system consists of a tandem array of a truncated human ßglobin gene and a CFP containing the peroxisomal targeting tripeptide SKL that is stably integrated into human U2OS cells. Upon transcriptional induction the active transcription site can be visualized via a series of repeated stem-loops that are specifically bound by an MS2 coat protein fused to GFP or RFP. Alternatively the transcription sites are detected by using FISH with probes for an intron. We show that neither proteasomes nor proteolytic foci are localized at active transcription sites. We propose a model where active proteasomes are organized in proteolytic foci occupying central nuclear positions. They may represent recycling centers that control transcription by recruiting factors for degradation.

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High-Contrast Single-Molecule Microscopy by Selective Focal Plane Illumination

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Wide-field single molecule fluorescence microscopy is a versatile tool for analyzing interactions in biophysical and biological systems in three dimensions without averaging over molecular ensembles. Known drawbacks of the technique are a high fluorescence background due to excitation of out-of-focus molecules, a limited axial resolution, and photobleaching. To overcome these problems we used a selective focal plane illumination instead of the conventional epi-illumination. A custom-designed objective lens (NA 0.33) was used to create a light sheet inside a water chamber with a thickness of 1.7 µm (FWHM at λ_{ex} = 543nm) perpendicular to the detection axis. Fluorescence light was collected by a water-dipping objective lens (60x, NA 1.0) and imaged onto an EMCCD-camera. By this means we constructed a simple optical sectioning microscope, which combines the contrast and high resolution of a confocal microscope with the speed and sensitivity of a video microscope. The lateral and axial resolution were 350 nm and 1.37µm, respectively (λ_{ex} = 633 nm; λ_{em} = 680 nm), similar as in confocal microscopy. In comparison to a conventional illumination we achieved a significantly improved signal-to-noise-ratio of > 110%. The optical sectioning capability of the instrument was demonstrated by acquisition of high-resolution 3D image stacks of polytene chromosomes in nuclei of *C. tentans* salivary gland cells. For demonstration of the new instrument's suitability to perform fluorescence experiments on the millisecond time scale we measured the diffusion coefficient of quantum dots (QD655) in buffer solution correctly to D = 17.5 $\mu m^2/s$. The new experimental setup eliminated major problems of single molecule microscopy in three-dimensionally extended geometries.

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Single molecule microscopy using total internal reflection (TIR)

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In single molecule fluorescence microscopy one often wants to reduce the fluorescence background emitted by fluorophores not located in the plane of interest. With total internal reflection fluorescence (TIRF) microscopy this is achieved by reflecting the light at the coverslip/probe interface (coverslip (glass) n = 1.51; sample (e.g. cell) n = 1.33), and thus generating an evanescent wave illuminating the sample. The intensity of the evanescent wave decays exponentially with a penetration depth of roughly half the wavelength of the incident light. We realised objective type TIRF using an NA 1.45 objective from Zeiss. Using this technique we characterized several red fluorescent dyes attached to the surface by a PEG-biotin-streptavidin system at the single molecule level. As a first biological application we studied the transport dynamics of single nuclear pore complexes.

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Determination of the architecture of the human kinetochore in living cells.

Daniela Hellwig

The kinetochore assembles at centromeric DNA and mediates chromosome attachment to microtubules during mitosis. Formation of this microtubule-binding interface is regulated by a network of proteins, the "mitotic checkpoint". Malfunction of this structure can lead to aneuploidy, cancer and aging.
The accurate organisation of the different sub-complexes (the "Next to CENP-A" NAC complex at the centromeric chromatin, the "CENP-A distal complex" CAD binding to the NAC, the hMis12 and Hec1 complexes) is still unknown. As a first step, we studied the interactions between these proteins by Yeast two hybrid assays. We use different live cell imaging techniques to analyse the constitution of the mechanical link between the inner kinetochore and the microtubules, which is an essential functional element of the kinetochore. Dynamic measurements (FRAP) and the determination of neighborhood relations (AB-FRET and FLIM) help to explain the architecture of the kinetochore and its build-up. Our results will lead to a model of kinetochore structure and will help to understand the function of this complex.

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Quantum dots as substrates for nuclear-cytoplasmic transport

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Nuclear-cytoplasmic transport of macromolecules is accomplished by the nuclear pore complex (NPC) - a transport machine imbedded in the nuclear envelope (NE). The NPC enables high selective translocation across the NE, known to be facilitated by the interaction of soluble transport receptors with the NPC's nucleoporins. However, detailed mechanisms and kinetics of the translocation still remain unknown. Single molecule fluorescence microscopy provides a direct observation of processes at the NPC with excellent spatial and time resolution. We use functionalized biocompatible quantum dots as transport substrates to investigate nuclear import in permeabilized cells at the single particle level. As bright and photostable probes, quantum dots yield an excellent localization precision (< 10 nm). This is of great importance when tracking the import complex through the approximately 100 nm long NPC.
Experiments with NTF2-functionalized quantum dots demonstrated that a specific interaction with the NPC can be achieved. We detected nuclear import of the smallest, green fluorescent quantum dots, however, their fluorescence is not bright enough to yield the required localization precision. The brighter red fluorescent quantum dots on the other hand could not pass the NPC, due to their larger stokes radius. Currently we are focussing on the preparation of smaller red fluorescent substrate-conjugated nanoprobes.

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Modification with Ferrocene Carboxylic Acid Facilitates the Membrane Crossing of [Leu5]-Enkephalin Derivatives in in-vitro Systems

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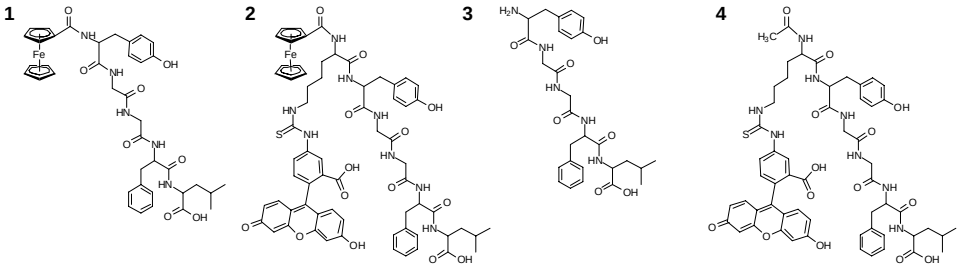
The modification of endogenous peptides with transition metal compounds is a growing field of interest, since the merge of the biological selectivity and activity of peptides and the unique characteristics of transition metal compounds can lead to improved properties. [Leu^5]-Enkephalin **3** is a pentapeptide which acts as a neurotransmitter in the central nervous system. It is also known as a potential vector for crossing the blood-brain-barrier (BBB).[1] Modifications, like glycosylation or lipidation, which do not alter the amino acid sequence are often used to enhance the permeation of Neuropeptides.[2] We introduce Ferrocenoylation as a new modification, which significantly enhances the crossing of Enkephalin-derivatives through biologicals barriers. In an in-vitro BBB-model **1** showed an improved permeation against **3**. Furthermore a considerable cellular uptake of **2** could be monitored by live cell fluorescence microscopy on two of three cancer cell lines, while **4** reached relevant uptake levels only at higher concentration. Also **1** was not cytotoxic on these cell lines. The compounds **1**-**4** were synthesised following the Fmoc-solid-phase-peptide-strategy [3], and purified by RP-HPLC before biological application. The results from the biological experiments correlated with measured log P_{oct}-values, which are a grade for lipophilicity.[4] A high lipophilicity favors the penetration of the BBB and cell membranes.[5] Thus Ferrocene or other organometallic compounds could be suitable to improve permeation of biologically active peptides in-vivo and lead to higher concentration and activity of relevant peptides by modification with Ferrocene carboxylic acid.



References

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4. D. J. Minick, J. H. Frenz, M. A. Patrick and D. A. Brent, *J. Med. Chem.,* **1988**, *31*, 1923-1933
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CELLULAR UPTAKE AND LOCALISATION OF METAL-PEPTIDE-BIOCONJUGATES

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Organometallic conjugates of cell-invasive peptides are proposed as interesting candidates for a future generation of novel cancer therapies since they are structurally unique compared to other classes of routinely used cytotoxic drugs. We have synthesised various metal compounds linked to peptides. As metal-containing moieties we have chosen ferrocene, cobaltocene and cobalt carbonyl. These peptide components should guide the metal into the cell and eventually into specific compartments The peptides we have chosen operate by receptor-mediated and also energy independent uptake mechanisms (cell penetrating peptides). Also, we have chosen signalling peptides for targeting of specific intracellular compartments. Incorporation of a fluorophore into these peptides has allowed us to image cell uptake and intracellular localisation by fluorescence and confocal microscopy (Leica TCS SP2 AOBS).
Peptides were synthesised using solid phase peptide synthesis with the Fmoc-protecting group strategy. The metallocenes were coupled to the N-terminus of the peptides directly on the solid phase. The cobalt carbonyl moiety was coupled to an alkyne-containing peptide in solution. The fluorophore fluorescein isothiocyanate (FITC) was coupled to the peptide by an additional lysine after removal of the Mtt protecting group. These bioconjugates were purified by RP-HPLC and characterised by NMR and IR spectroscopy and mass spectrometry.
The evaluation of these peptides in cell culture will be presented. Cellular uptake, and the effect of the metal was investigated by comparison of metallocene-peptide conjugates to acetylated peptides using fluorescence microscopy. Intracellular fate was examined by co-localisation studies using DRAQ 5, Lysotracker Red and FM 4-64 for staining the nucleus, lysosomes or endosomes.

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Subnuclear positioning of protein aggregates

Lena Singer and Anna von Mikecz

Protein aggregates and nuclear inclusions containing components of the ubiquitin-proteasome system, expanded polyglutamine proteins, and transcriptional coactivators characterize cellular responses to stress and are hallmarks of neurodegenerative diseases. A nanoparticle-based method enables controlled induction of protein aggregates of these endogenous marker proteins and topoisomerase I. Since topoisomerase I is a specific and sensitive marker for nanoparticle- induced protein aggregates it was used for further characterization of the aggregates. Immunofluorescence experiments were performed with marker proteins of prominent nuclear structures such as the nucleolus, the nuclear envelope, PML- bodies and speckles and their position correlated to topoisomerase I aggregates were determined by means of distance measurements. We show that topoisomerase I aggregates are juxtaposed to the nucleolus (52,8%) and PML- bodies (22,9%) and partially (42%) or entirely (20%) colocalize with speckles.
These results suggest that nanoparticles disturb the close structure and function relationship in the nucleus by sequestration of nuclear proteins in aggregates and impairment of functions such as transcription.

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C. elegans is a target of nanoparticles

Adam Pluskota, Eva Horzowski, Olaf Bossinger and Anna von Mikecz

In the last decade nanotechnology became an impotant area of science and their use in technical applications is increasing. Currently, nanoparticles are produced on large scale and thus can be found in consumer products, including pharmaceuticals and food. Despite of their broad distribution, the understanding on how nanoparticles interact with biological systems is limited. The work presented here provides first results about the effects of silica and polystyrene nanoparticles on the modelorganism *Caenorhabitis elegans*. Fluorescently labelled polystyrene nanoparticles translocated into different tissues of the hermaphrodite and were also found in the developing embryos. Fluorescently-labelled silica nanoparticles were only detectable in the lumen of the gut. Interestingly, nanoparticle treatment did not significantly alter the mean lifespan of wildtype nematodes.

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Polystyrene-nanoparticles: Cellular uptake and effects on the cell nucleus

Insa Larson and Anna von Mikecz

Nanoparticles (NPs) have a size between 1 and 100nm. It was shown that NPs enter the cell and induce changes in cellular structure and function. In my diploma thesis I analyzed the cellular uptake and the cell biological effects of fluorescently labelled polystyrene-NPs. We show by means of living cell observation that polystyrene-NPs enter cells within minutes. In the cytoplasm NPs distribute in a tubular-like pattern. Since cell viability was uneffected we conculde that polystyrene-NPs are not cytotoxic. To analyze if polystyrene NPs translocate to known cellular compartments, different living cell dyes were used. Interestingly, polystyrene-NPs do not colocalize with lysosomes, the endoplasmic reticulum, the golgi complex or chromatin. Partial colocalization was observed with the mitochondria specific dye mitotracker. In contrast to SiO_2-NPs that induce protein aggregates consisting of topoisomerase I in the nucleoplasm (Chen and von Mikecz, 2005; Chen et al., 2008), we did not observe any alterations of nuclear structure after exposure of cells with polystyrene-NPs.
In summary, cellular responses to different NPs seem to depend on specific nanoparticle properties.

Literature:

Chen, M. & von Mikecz, A. 2005. Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO_2 nanoparticles. *Experimental cell research*, **305** (1), S. 51–62.

Chen, M., Singer, L., Scharf, A. & von Mikecz, A. 2008. Nuclear polyglutamine-containing protein aggregates as active proteolytic centers. *The Journal of cell biology*, **180** (4), S. 697–704.

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

Tobis Ulbricht

Regulated positioning of nuclear structures and specific genes within the cell nucleus is essential for efficient gene expression.
PML nuclear bodies (NBs) are subnuclear structures present in most mammalian cells. They are associated with a multiplicity of proteins (> 78) depending on cell type, cell cycle state and exogenous conditions. PML NBs appear to be involved in key nuclear pathways by titration, modification and compartmentalization of specific factors.

These nuclear structures predominantly localize at gene-rich and trasncriptionally active genomic loci, particularly at the major histocompatibility complex class II (MHC II) gene cluster on 6p21.3. We have shown previously that this non-random association can be increased by interferon gamma (IFNγ) in different cell lines of non-hematopoietic origin. It is unclear, however, if PML NBs are functionally involved in the expression of MHC II genes.

We analyzed the nuclear partitioning of specific MHC II transcription factors and showed that a subset of these (RFXb, RFXap, CIITA) indeed accumulate at PML NBs. Furthermore siRNA mediated knock down of the PML protein results in the loss of the PML NBs as well as in a significant decrease in expression of HLA molecules. This decrease might be caused by the reduction of the MHC II transactivator (CIITA) protein level after PML knock down. These data suggest that PML NBs may be directly involved in the regulation of MHC II genes by sequestration and/or stabilization of specific MHC II transcription factors.

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Chromatin alterations during Cellular Senescence

Karolin Klement

Senescence is a permanent cell cycle arrest due to the replicative exhaustion of cultured normal diploid cells. Primary human fibroblasts can pass 30-50 population doublings until the senescent phenotype establishes. In vivo, cellular senescence constitutes a cell cycle arrest that limits the proliferation of damaged cells and is therefore considered as a tumor suppressor pathway.
Different distinct senescence markers exist, such as irreversible G1-arrest, flattened and enlarged morphology and an altered gene expression. Senescent cells are still viable and metabolically active, specifically express senescence-associated (SA)-β-galactosidase and develop a high level of p16. Senescent cells in vivo and cultured cells develop a specialized form of heterochromatin termed "SAHF" (senescence associated heterochromatin foci).The mechanism of SAHF formation is not understood. Different chromatin proteins have been suggested to play a functional role in SAHF formation and maintenance, e.g. HMGA1/2, macroH2A, HIRA and ASF1a. Our goal is to further elucidate the molecular mechanisms that drive SAHF assembly and to better understand the role of SAHF in establishing the senescence phenotype. In this project we want to investigate if SAHF formation is a key element of cellular senescence and which additional proteins contribute to SAHF formation. To answer this question we developed a drug-based assay to monitor the senescence pathway in primary human fibroblasts. Based on this assay and on the conventional replicative senescence approach we perform differential mass spectrometry analyses of chromatin from young vs. senescent cells in order to identify new senescence-specific chromatin alterations. Furthermore we investigate how SAHF components cooperate to assemble this specialized structure using fluorescence live cell techniques. First results will be presented.

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Senescing human cells accumulate PML at unrepairable DNA double strand breaks

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PML nuclear bodies are common subnuclear structures in mammalian cell nuclei. Their major structural component is the promyelocytic leuemia (PML) protein. The PML protein and the PML nuclear bodies appear to be involved in DNA repair, apoptosis and senescence, but little is known about how PML mediates these functions.

Cellular senescence constitutes a cell cycle arrest that limits the proliferation of damaged cells and is considered as a cellular counterpart of organismal aging. Senescence can be induced by dysfunctional telomeres, DNA damage and oncogenic or oxidative stress. The finding that DNA damage sites accumulate in senescening cells specificially indicates DNA double-strand breaks (DNA DSBs) and their repair as critical factors in the aging process.

We have analyzed the spatial relationship between PML nuclear bodies and DSBs during replicative and premature senescence. We demonstrate that PML nuclear bodies are not associated with sites of successful DSB repair, but only with unrepaired DNA DSBs after severe damage. We could further show that the PML protein is not required for successful DNA repair after γ-irradiation of human and murine cells. We also observed that PML specifically associates with accumulated DNA damage sites in senescent cells. These observations suggest a signaling or mediator function for PML at unrepairable DNA double-strand breaks in aging human cells.

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Structure and function of the inner human kinetochore

Christian Weber

The vertebrate kinetochore is a highly conserved complex structure, consisting of an inner and an outer plate. It mediates the binding of spindle microtubules to the chromosomes, thus being essential for accurate chromosome segregation. The inner kinetochore is composed of several protein complexes. Their neighbourhood relations and interactions will yield the structural composition. Currently these proteins are analysed in our group by various techniques: yeast two hybrid, fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS) and fluorescence resonance energy transfer (FRET, FLIM). Because these results do not provide information about the stoichiometry and the three-dimensional structure, Strep-tagged CENP-A and CENP-C associated complexes were purified and analysed with transmission electron microscopy. The studies will be extended to cryo electron microscopy, atomic force microscopy and mass spectrometry. Taken together, the data about structure, stoichiometry, dynamics and neighbourhood relations will lead to the three-dimensional structure of the inner kinetochore protein complex. This is a key step in order to understand its function.

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Alternative Lengthening of Telomeres in a Telomerase negative osteosarcoma cell line

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The maintenance of telomeres is crucial for the unlimited cell proliferation of cancer cells and is achieved in most cases by telomerase-reactivation. However, there are about 15% of cancer cells that use a different mechanism called alternative lengthening of telomeres (ALT), which is based on DNA recombination and repair. ALT positive cells are characterized by a great heterogeneity in their telomere repeat lengths and the presence of ALT associated PML bodies referred to as APBs. Many molecular details of the ALT mechanism are currently unknown.
For detecting specific telomeres *in vivo* in the ALT positive osteosarcoma cell line U2OS, we use the *lacO*/LacI-XFP system in a cell clone that has 3 *lacO* – arrays integrated in the telomeres of 3 different chromosomes. These sites were visualized in time-lapse imaging experiments via bound fusion proteins of LacI with a fluorescence protein. The mobilities of the labelled telomeres were characterized and correlated with the corresponding telomere repeat length as determined from a TRF2-GFP signal at these loci. The results indicate that shortening of the telomere repeats leads to an increase in telomere mobility and are discusses in terms of a model for the ALT mechanism.

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Different methods for distance definitions inside of the human cell nucleus

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Chromosomal aberrations constitute a prominent hallmark of human cancers. Over a broad dose range (down to less than 100mGy), ionizing radiation is a potent inducer of chromosomal aberrations. Various studies have indicated that proximity of chromosomal territories (CTs) within interphase nuclei has a strong impact on formation of chromosome exchange aberrations, both spontaneously and after ionizing radiation. Consequently, chromosomal aberrations might differ for various tissues reflecting the tissue specific relative positioning of CTs. Novel insights have been gained on CT arrangements in fibroblasts that contrast lymphocytes: in fibroblasts CT arrangements correlate mainly with the size, in human lymphocytes with the gene density. To quantify possible effects of the different relative positioning of CTs on the induction of chromosomal aberrations, in the present study relative positions of CTs #1,#19,#4,#18 were evaluated in XP23 fibroblast human cell nuclei. The distance evaluations were compared then with respective simulated model calculations of human fibroblast nuclei. Virtual low LET radiation aberration estimates, executed for these modelled human fibroblasts, were applied to quantify differences to human lymphocyte nuclei.
Distance distributions can be performed in different ways: The interest can be focused on the distance from or to the whole CT-Volume, or to the distance from or to its border or from or to its border only. Also, two ways of calculations are applied: Either one measures the distance distribution from or to every voxel of a given object, or only the shortest distance of one object to the other. The principal approaches to perform distance measurements and respective results are presented.

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Structure resolution in localization microscopy

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Different applications of localization microscopy demonstrated the usefulness of this new method for nanoscale imaging of various fluorescent labeled structures. While the very first localization experiments based on Spectral Precision Distance Microscopy used markers of different colors for the spatial separation of their signals, in recent techniques the object discrimination is done in time by stochastic activation or other methods. Regarding the fact that the localization of single light emitting molecules is a statistical process the demand for connection between the determined localization accuracy and spatial resolution is obvious. This coherence can be shown in simulations. The results demonstrate the possibilities of localization microscopy in structure reproduction.

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PNA COMBO-FISH, Molecular labelling with specific oligo-probe sets

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COMBO-FISH (**Comb**inatorial **O**ligo **F**luorescence **i**n **S**itu **H**ybridisation) is a novel technique that has been used to specifically label genome targets in cell nuclei under vital conditions. Novel applications of COMBO-FISH labels are under development to analyze the genome nano-architecture of breakpoint regions (e.g. *abl*; *bcr* ; *Ryr2*) and tumor genes (e.g. *Her2/neu* ; *GRB7* ; *c-myc*) in fixed cell nuclei as well as in vital cells. Studies of the arrangement of the target sites by spinning disc confocal, laser scanning microscopy and gene compaction by Spatially Modulated Illumination (SMI) microscopy in combination with COMBO-FISH offers new perspectives in tumor diagnosis, risk estimate and tumor therapy control.
COMBinatorial Oligo (COMBO)-FISH is working with computer selected, specifically co-localizing combinations of oligo-nucleotide probes which bind either triple-helically to the DNA double strand via hoogsteen-bondings or to the complementary DNA via Watson-Crick-bondings. This helps to maintain the nano-structure and native compaction of genes. In addition, this method has the potential to label cell nuclei under vital cell conditions after probe transfer by microinjection or other established procedures (Magnet Assisted Transfection – MATra [IBA GmbH, Göttingen]).
In this study we show that COMBO-FISH is also feasible with PNA (**P**eptide **N**ucleic **A**cids) probe sets that are labelled with different fluorophores. Proof-of-principle experiments were done with a repetitive PNA oligomere that hybridize in the subregion of centromere 9. The PNA oligomeres were differently labelled with standard fluorochromes such as Alexa Fluor 488, Oregon Green 488, Carboxytetramethylrhodamine (TAMRA) or Texas Red (Invitrogen).
After the establishment of COMBO-FISH with these repetitive PNA oligomeres a probe set specific for a distinct gene region was synthesized. This probe set consists of 19 computer selected homopyrimidine oligomeres (17-22 bp) which co-localize with high specificity in the *Her2/neu* gene region on the long arm of chromosome 17 (17q 12-21.32).
The *Her2/neu* (human epidermal growth factor 2) gene is a protooncogene that mostly undergoes a gene amplification in breast cancer cells and plays an important role in the regulation of cell growth and proliferation. The COMBO-FISH experiments were first of all realised on methanol/acetic acid fixed peripheral blood lymphocytes obtained from a healthy donor.

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Light optical nanoscopy of genome structure in mouse mammary cancer tissue

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Transgenic mouse models have been widely used to study the role of a particular gene in oncogenesis. In collaboration with the Jackson Laboratory (Bar Harbor, Maine), we have established a biophysical platform to study mammary cancer related nanostructural gene abnormalities in transgenic mouse.
SV40Tag (simian virus 40 large tumor antigen) is known to be able to transform cells into neoplastic phenotype and is highly oncogenic. By fusing the WAP (whey acid protein) gene regulatory region and the SV40Tag gene coding region, the viral oncogene SV40Tag can be targeted to the mammary epithelium of lactating females, creating a mouse model for mammary cancer, which is histopathologically comparable to the case of human. By labelling the gene region and the relative chromosome 11 using Fluorescent in situ Hybridization (FISH) method, the changes of the gene extension and its relative position in the chromosome territory during oncogenesis can be measured by Spatially Modulated Illumination (SMI) microscopy and other novel laseroptical precision microscopy methods.
So far, the gene and its chromosome territory has been successfully labelled in mouse fibroblast cells and 500nm cryosections of mouse mammary tissue. The measured size of the SV40Tag gene in mouse fibroblast cell by SMI microscopy is 93.4±25nm (in the range of the calculation and simulation results), which presents the gene's extension in its active state. Statistics of the gene state in cryosections is on its way.

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Receptor clusters in *E. coli* measured with 4Pi- and SMI-Microscopy

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Chemotaxis in *Escherichia coli* is one of the most thoroughly studied model systems for signal transduction. Receptor-kinase complexes, organized in clusters at the cell poles, sense chemoeffector stimuli and transmit signals to flagellar motors by phosphorylation of a diffusible response regulator protein. Recent advances in computer modeling and in quantitative experimental analysis suggest that cooperative protein interactions in receptor clusters play a crucial role in the signal processing during bacterial chemotaxis.
The presented measurements were done with a commercial confocal laserscanning 4Pi-microscope (Leica TCS 4Pi) as well as with a SMI-microscope (SMI: Spatially Modulated Illumination) developed at the Kirchhoff-Institute for Physics.
In both microscopical methods the sample is coherently illuminated through two opposing microscope objectives. In 4Pi microscopy, constructive interference of the counter-propagating spherical wavefronts narrows the main focal maximum of the excitation light in the z-direction. This leads to a 3- to 7-fold improvement of the axial resolution compared with conventional confocal microscopy.
The SMI-microscope is a widefield fluorescence microscope with structured illumination in the axial direction. The interference of two counter-propagating collimated laser beams creates a standing wave field of $\cos^2$ form. Although this is not directly improving optical resolution, with some assumptions about object form it is possible to get structural information about the object far below the resolution limit. In this case, SMI-microscopy allowed quantitative measurements of the size of receptor clusters in a resolution range beyond conventional confocal microscopy.

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Functional Implications of Myosin IC in Cytoskeleton Reorganization

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The ubiquitiously expressed class I myosins are single headed, non-filamentous motorproteins that play a role in various motile processes. Here, we investigated the role of *Dictyostelium discoideum* myosin 1C in the reorganization of the cytoskeleton.
Myosin-IC appears to act in the formation of cell surface extensions, such as endocytotic cups. Since myosin-IC knockout cells display no pronounced phenotypic changes in phagocytosis or pinocytosis, we assume that myosin-1C function in endocytosis can be taken over by other class I myosins, like myosin-1D or myosin-1E.
In addition, our results suggest a role of myosin-1C at the mitotic spindle. This is implicated by our finding that the protein relocalizes at the onset of mitosis and predominantly associates with the mitotic spindle from prophase to telophase. In ultracentrifugation assays we measured that the myosin-1C tail domain tightly associates with microtubules (K_d ~1 µM) and F-actin (K_d >10 µM). Fluorescence and TIRF microscopy experiments showed that myosin-1C tail crosslinks these two cytoskeletal elements and promotes microtubule bundling. It also strongly protects microtubules from depolymerisation with an IC_{50} of about 25 nM. Consistently, we detected by the use of confocal microscopy that myosin-1C knockout cells exhibit a reduced microtubules density in the mitotic spindle and a reduced astral tubule size.
We conclude that myosin-IC during mitosis functions in spindle orientation and positioning. Additionally, myosin-IC motor activity may be required for anchoring of the mitotic spindle.

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MAPkinases and their role in regulation of protein SUMOylation

Ratnesh Kumar Srivastav

SUMO (Small ubiquitin like modifier) is ~100 amino acid long protein which conjugate post-translationally to substrate proteins in transient and reversible manner. Conjugation of SUMO proteins to target proteins requires three different kinds of enzymes, E1, E2 and E3. E1 enzyme conjugates to mature SUMO in ATP dependent manner and form thioester bond. E2 enzyme or SUMO conjugating enzyme (Ubc9) interact with loaded E1 enzyme which transfer activated, mature SUMO protein to Ubc9. Ubc9 directly interact with substrate protein or it`s interaction with substrate protein is directed by target specific ligases (E3). Despite of constitutive expression of SUMO1 there is only a little fraction(1-2%) of any protein SUMOylated. Although a number of proteins have been characterized which undergo SUMOylation the function of this SUMOylated fraction is still obscure.
We are interested in MAPkinases dependent regulation of protein SUMOylation. For this we have identify a number of proteins which show enhance SUMOylation when co-express with MEKK1ca. Now we want to study effect of phosphorylation dependent enhanced SUMOylation on the substrate protein. For this we want to use knock down strategy and new microscopic techniques .

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Raster Image Correlation Spectroscopy (RISC)

Eugen Baumgart

In contrast to conventional wide field illumination, in confocal laser scanning microscopy the image is acquired consecutively pixel by pixel, either in raster scanning, line scanning or circular scanning mode. This scanning approach thereby includes otherwise hidden spatiotemporal information, which can be used to obtain additional insight into the systems dynamics, like the diffusion coefficient.
The RICS technique extends Fluorescence Correlation Spectroscopy from a stationary, illuminated volume to a moving beam and makes the features of FCS accessible on commercial confocal LSMs, which are widely used in current life science research.
The basic principle of RICS is to calculate the temporal and spatial correlation between adjacent pixels, lines and frames and thereby providing access to three different time scales, on which dynamic processes can occur.
We use the SimFCS program, developed by Enrico Gratton and coworkers at the University of California, Irvine, to simulate LSM images of particles with a defined diffusion constant and use the implemented correlation routines to fit the respective correlation functions. From the fit parameters, the diffusion constants can be derived again and compared to experimentally measured values.
Our aim is to augment our commercial laser scanning microscope (Zeiss LSM 510 Meta) with the RICS technology to allow measuring diffusion constants of fluorescent particles *in vitro* and also *in vivo*. Results will be directly compared with experiments using Single-Molecule-Microscopy- and Single-Particle-Tracking routines developed in our research group.

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Structure and function of the mitotic checkpoint protein complexes

Volkker Döring

The anaphase promoting complex/ cyclosome (APC/C) is a stable multiprotein complex that triggers the progression from metaphase to anaphase during mitosis. This E3-Ligase consists of at least 12 subunits and initiates sister chromatid-separation by ubiquitinylation of securin, the repressor of separase. Seperase becomes free and is then able to degrade the sister chromatides connecting cohesion. In case of missing or improper spindle-attachment of only one kinetochore to the spindle apparatus a stop signal is generated by the spindle assembly checkpoint. This checkpoint is a surveillance mechanism that inhibits the APC/C by assembly of the mitotic checkpoint complex (MCC). The MCC is formed by BubR1, Bub3, Mad2 and Cdc20. Only when all kinetochores are properly attached to the spindle microtubules, the MCC becomes deactivated and Cdc20, a potent mitotic activator of the APC/C, can bind and activate the APC/C. Failure in this system results in premature sister chromatid separation which in turn may lead to aneuploidy, cellular senescence or even cancer. Although the architecture of the human and yeast APC/C is elucidated, current knowledge of the interaction between the MCC, the outer kinetochore and the APC/C is limited. Also little is known about the time resolved distribution of those complexes. We therefore are interested in the identification of the interaction partners between MCC, the outer kinetochore and the APC/C. Also we will study dynamics and molecular interactions. For our *in vivo* analysis, we started to localize fluorescence-tagged proteins of the MCC and APC/C respectively in living human cells. To investigate stability, composition, interaction and the dynamics of all the proteins, we will perform FRAP, FRET, FCS and FLIM analysis. Therefore we tagged each protein with C- and N-terminally to various fluorescence proteins (GFP, YFP, Cerulean, mCherry, mRFP). Beside our *in vivo* studies, we want to analyse protein-protein-interactions by *in vitro* analysis. Therefore we also tagged each protein of the MCC and the APC/C with Y2H expression-tags. The identification of the interaction partners of the MCC and the APC/C and the time resolved localisation of all components may lead to a better understanding of the complex system that grants a correct distribution of the sister chromatides to each daughter cell during mitosis.

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Segregation and Cohesion of the *ter* region in the model bacterium *E. coli*.

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We are interested in bacterial chromosome organization and segregation. We are using *E. coli* to study the late steps of the cell cycle linking replication termination, chromosome segregation and cell division. These processes must be tightly coordinated in order to ensure proper cell proliferation. One major actor is the motor protein FtsK which plays a role in the late steps of chromosome segregation as well as in cell division. FtsK is anchored at the division septum, is able to read chromosome polarity and to pump DNA. Notably, FtsK activity is required for resolving chromosome dimers than can occur during circular chromosome replication. We are currently studying the role of FtsK (and other factors) in the segregation and cohesion of the *ter(minus)* region of the chromosome, being the last replicated and segregated before cytokinesis. To this end, we are using genetic and cytological methods to gain insights into the peculiar structure and processing of the ter region during cell division.
We show that FtsK acts in a ~400 kb region around the dif natural position. This FtsK domain can be uncoupled from the zone where replication terminates. We examined the segregation dynamics of chromosomal loci belonging to the FtsK domain (using the bacteriophage P1 ParB-GFP/ParS system). Further analyses of segregation using 3D confocal microscopy should improve our understanding of the final chromosome segregation events.

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Further development of a two photon setup to activate and localize single molecules

Manuel Gunkel

Topical biological questions require information about structures which are only a few tens of nanometers in size. These structures cannot, however, be resolved using conventional light microscopy. One method of obtaining this information is localisation microscopy, in which the position of fluorescent objects can be determined with an accuracy very much better than the resolution limit. When using photo-activatable molecules it is possible to separately activate individual fluorophores over multiple cycles. The single fluorophores can thus be independently localised. Current techniques applying this principle such as Photo Activated Localisation Microscopy (PALM) achieve an effective lateral resolution in the range of a few tens of nanometers. One method of implementing 3D localisation is to restrict the activation to a small region along the optical axis by using a focused laser beam and the 2-photon effect to activate the fluorophores. Single molecule preparations of both standard and caged fluorescein were observed. The results show that, with the current setup, 50.000 photon counts could be detected, facilitating a two dimensional localisation accuracy of 2nm. Experiments with cells were performed in order to verify the 2-photon activation from single molecules in the cell occurred within optical sections.

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