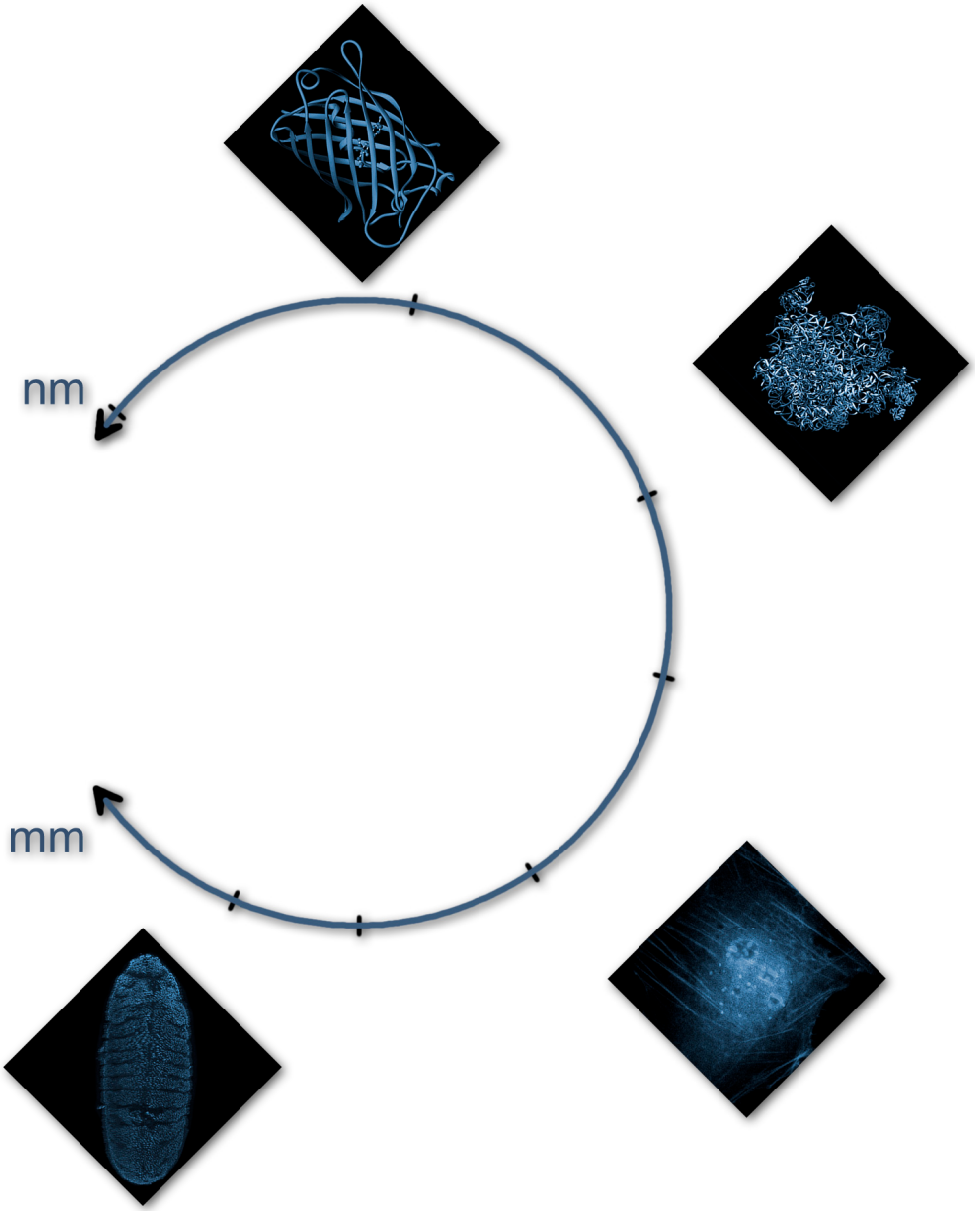
International Symposium

Optical Analysis of Biomolecular Machines

July 13th - 16th, 2006

Max Delbrück Center for Molecular Medicine, Berlin, Germany



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Symposium Outline

The aim of this international symposium to be held in the context of the DFG Priority Programme “Optical Analysis of Supramolecular Biological complexes” (DFG SPP1128; coordinator C.Cremer/Heidelberg) is to discuss the novel developments and applications of optical analyses of biological nanostructures, their components and their underlying dynamics. The emphasis will be in joining together the methodological developments with the biological applications within each and every session; wherever possible, computer simulations and kinetic modeling will be included.

Organizers:

M. Cristina Cardoso (Berlin)

www.mdc-berlin.de/cardosolab

Christoph Cremer (Heidelberg)

www.kip.uni-heidelberg.de/AG_Cremer/english/index.html

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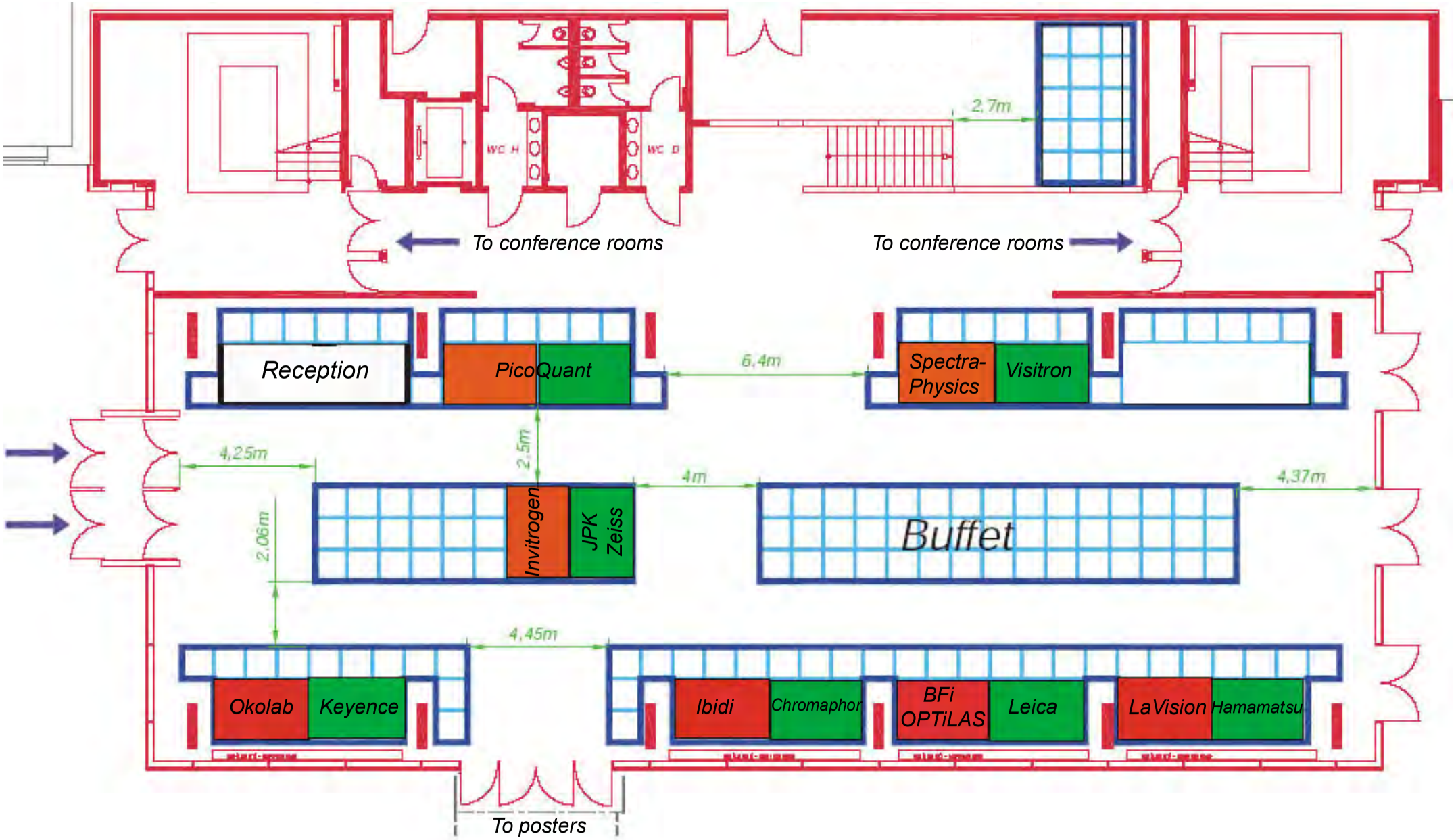
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General Information

Conference Office (MDC.C)

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Opening hours:

Thursday, June 13	08:00 - 21:30	Friday, June 14	08:00 - 19:00
Saturday, June 15	08:00 - 19:00	Sunday, June 16	08:00 - 19:00

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Services

Internet access
During the entire conference computers with permanent internet access are at your disposal in the MDC.C, Dendrit I on the 3rd floor.

Taxi
Taxi can be organized via the conference office or call: 0800 44 33 222

Public Transportation

Bus shuttles will be provided twice a day between hotels and the meeting place. Busses number 351, 158 and 150 go every ten minutes between S-Bahnhof Buch and the campus. For a detailed timetable ask at the conference office.

Contents

Program

Thursday, July 13th, 2006

14	*Registration* *Set up Posters*
17:15	*Welcome* *Walter Birchmeier and M. Cristina Cardoso*

Keynote Lecture

17:30-18:30	Roger Kornberg: Imaging the Eukaryotic Gene Transcription Machine

Microscopic imaging at the one nanometer scale

18:45-19:15	David P. Bazett-Jones: Interactions between chromatin and PML nuclear bodies: insights from fluorescence microscopy and electron spectroscopic imaging
19:15-19:45	Christian M. T. Spahn: Single particle cryo-EM of the translational apparatus
19:45-20:00	Vladan Lucic: Cryo-Electron Tomography of Neuronal Processes and Synapses
20:00	*Welcome Reception*
21:30	*Bus departure*

Friday, July 14th, 2006

Light microscopy from the nanometer to the micrometer scale I

9:00-9:30	Rainer Heintzmann: High Resolution Structured Illumination Microscopy
9:30-10:00	Udo J. Birk: Nanosizing and Structure Analysis of Gene Domains and Protein Complexes
10:00-10:20	Lothar Schermelleh: Ultra-high resolution light microscopy using structured illumination
10:20-10:40	Jörg Enderlein: Dynamic Saturation Optical Microscopy

Coffee Break

Program

Molecular Labelling Workshop

11:15 -11:45	Konstantin A. Lukyanov: Fluorescent proteins: a toolkit from nature
11:45-12:05	Alice Ting: New reporters for imaging protein trafficking and function in living cells
12:05-12:25	Carsten Schultz: Imaging of an enzyme-substrate complex in living cells
12:25-12:40	Andrea Glatzel: Fluorescent Live Cell Imaging and Tracing The challenge of long term live cell studies
12:40-12:55	Dorothea Lorenz: Coumarinylmethyl esters for ultrafast release of high concentrations of cyclic nucleotides upon one- and two-photon photolysis
13:00	*Lunch (Barbecue / weather permitting)*
14–16	**Poster session I / Industrial Exhibition**
15:00	*Guided Tour "Historical Microscope Collection"* *Helmut Kettenmann*

Coffee Break

Light microscopy from the nanometer to the micrometer scale II

16:30-17:00	Stefan W. Hell: Far-field fluorescence microscopy at the nanoscale: breaking Abbe's diffraction barrier by the RESOLFT concept
17:00-17:20	Steffen Rüttinger: Quantitative single pair FRET by pulsed interleaved excitation and fluorescence correlation spectroscopy
17:20-17:40	Frank Schleifenbaum: Combining spectroscopic methods for investigation of single autofluorescent proteins on the nanoscale
18.00	*Bus transfer to Conference Dinner* *Thoru Pederson: Alfred Nobel, and his Prizes*

Saturday, July 15th, 2006

Program

Light microscopy from the micrometer to the millimeter scale

9:00-9:30	Scott E. Fraser: Four-Dimensional Imaging of Embryonic Tissue Dynamics
9:30-10:00	Ernst H. K. Stelzer: Selective Plane Illumination Microscopy: Life sciences require the third dimension
10:00-10:20	Timm Schroeder: Tracking of stem cell behavior at the single cell level: new tools for old questions
10:20-10:40	Sylvia Münter: Quantitative analysis of Plasmodium sporozoites motility

Coffee Break

Dynamic analysis in vivo by light microscopy I

11:15-11:45	Robert H. Singer: Following the Synthesis and Travels of Single mRNAs in Living Cells
11.45-12:15	Rainer Pepperkok: Studying membrane turnover and complex formation of vesicular coat proteins in living cells
12:15-12:35	Stefanie Weidtkamp-Peters: Centromere assembly through highly immobile and some mobile foundation kinetochore proteins
12:35-12:55	Günter Gerisch: The 3-dimensional organization of free-running actin waves
13:00	*Lunch (Buffet)*
14-15	**Poster session II / Industrial Exhibition**
14:00	*Guided Tour "75 Years of Research in Berlin-Buch" Annett Krause*

Dynamic analysis in vivo by light microscopy II

15:00-15:15	Jeffrey H. Stear: Analysis of XMAP215-mediated microtubule growth by TIRF microscopy
15:15-15:30	Sabine M. Görisch: Replication fork progression in the absence of processive DNA synthesis
15:30-15:45	Mini Jose: Visualisation of interactions in living cells using FLIM and FRET
15:45-16:00	David Grünwald: Single molecule biochemistry in living cells and distribution in living cells

Coffee Break

Novel Approaches in Microscopy Workshop

16:30-17:00	Jason R. Swedlow: Functional Studies of the Mitotic Spindle in Cells and Tissues
17:00-17:30	Erik Manders: How to deal with photobleaching and phototoxicity in life-cell imaging
17:30-17:45	Kate Poole: Fully integrated light and atomic force microscopy to study cellular structure and adhesion
17:45–18.00	Volker Buschmann: Time-Resolved Confocal Fluorescence Microscopy with Single Molecule Sensitivity: Novel Technical Features and Applications for FLIM, FRET and FCS
18:00-18:15	Ulrich Kubitscheck: Single molecule microscopy using focal plane illumination
18:30-19:00	*Piano Recital*
19:15-21:00	SPP PI Meeting (preparation of third funding period)

Last Chance for Poster Award Voting!

Sunday, July 16th, 2006

Dynamic analysis in vivo by light microscopy III

9:00-9:30	M. Carmo-Fonseca: Using photobleaching microscopy to dissect spliceosome dynamics in the nucleus
9:30-10:00	Jean-Baptiste Sibarita: Tools for the analysis of cellular and intra-cellular activity at high spatial and temporal resolution
10:00-10:20	Christophe Zimmer: Building high resolution maps of gene positions in yeast nuclei by automated 3D image analysis
10:20-10:40	Jurek W. Dobrucki: Imaging of collagen in live tissues, using a new fluorescent probe PhF

Coffee Break

Correlation of Light Microscopy and Kinetic Modelling

11:15-11:45	Reinhart Heinrich: Mathematical modeling of cellular reaction networks with special focus on nucleotide excision repair
11:45-12:15	Stan Gorski: Dynamics of Transcriptional Regulation in Living Cells
12:15-12:40	Yaron Shav-Tal: Analyzing the transcriptional kinetics of an active gene in living cells

12:40-13:00	Gregor Kreth: Dynamic simulation of active and inactive chromatin domains
13:00	*Poster award ceremony and closing remarks* Christoph Cremer
13:30	*Lunch, End of the meeting*

Imaging the Eukaryotic Gene Transcription Machine

Roger Kornberg

Stanford University Medical School, Structural Biology, Fairchild Building, 299 Campus Dr., Stanford, CA 94305, USA

RNA polymerase II is the central molecule of eukaryotic gene expression. The intricate regulation of RNA polymerase II transcription underlies cell differentiation and development. Research of the past three decades has revealed the entire RNA polymerase II transcription machinery and has illuminated its mechanisms and its relationships to human disease.

Biochemical studies have identified nearly 60 polypeptides required for RNA polymerase II transcription, as well as components of chromatin-remodeling (SWI/SNF, RSC) and chromatin-modifying complexes. The required polypeptides comprise the 12-subunit RNA polymerase II, multiple "general transcription factors", and a 21-subunit "Mediator". The general transcription factors are responsible for promoter recognition and for melting the DNA template for the initiation of transcription. Mediator makes the key connection between enhancers and promoters. It transduces regulatory information from activator and repressor proteins to RNA polymerase II. There is a perfect one-to-one correspondence and high degree of sequence homology between all subunits of yeast and human polymerases and general transcription factors. Mediator exhibits a high degree of evolutionary conservation as well.

Structural studies of the RNA polymerase II transcription machinery began with electron microscope analysis of two-dimensional protein crystals formed on lipid layers. This led to the derivation of a 10-subunit form of RNA polymerase II especially conducive to crystallization, and to the use of two-dimensional crystals as seeds for the growth of large single crystals for X-ray analysis. The large size of the polymerase, over half a million Daltons, presented unusual technical difficulties, that were overcome by crystal growth and maintenance in anaerobic conditions, crystal shrinkage, and the use of nonstandard heavy atom derivatives. Structure determination at 2.8 Angstroms resolution from multiple crystal forms revealed a division of the polymerase in massive mobile elements, surrounding a nucleic-acid-binding cleft.

RNA polymerase II was also crystallized in the form of an actively transcribing complex, containing template DNA and product RNA. The structure of this complex was solved by molecular replacement at 3.3 Angstroms resolution, revealing the DNA entering and unwinding in the active center cleft. Nine base pairs of DNA-RNA hybrid could be seen extending from the active center at nearly right angles to the entering DNA. Protein-nucleic acid contacts help explain DNA and RNA strand separation, the specificity of RNA synthesis, and RNA and DNA translocation during transcription elongation.

Finally, electron microscopy and X-ray crystallography of RNA polymerase II in complexes with general transcription factors and Mediator have begun to reveal the architecture of the entire, 3 million molecular weight, transcription initiation complex. Insights from this work include the basis for promoter recognition, transcription initiation, and "promoter escape."

Interactions between chromatin and PML nuclear bodies: insights from fluorescence microscopy and electron spectroscopic imaging

G. Dellaire, H. Dehghani, R. Ching, C.H. Eskiw, D.P. Bazett-Jones

Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada

Our laboratory applies a variety of imaging technologies with standard biochemical methods to study aspects of gene activity. In particular, electron spectroscopic imaging has provided insights into nuclear organisation because of the ability to distinguish protein-based structures from DNA- and RNA-based complexes. Delineation of these biochemical components is made possible through detection and mapping of elements such as phosphorus and nitrogen [1-3]. The promyelocytic leukaemia nuclear body (PML NB) is a good example of a sub-organelle where specific nuclear functions may be compartmentalized, though how it functions remains an unanswered, but a fundamental question. A prevailing model of the PML NB is that it represents an accumulation of multi-subunit macromolecular complexes, some of which may carry out post-translational modifications of regulatory molecules, including p53 as a prime example. By studying the structure, biochemical composition and dynamics, we have extended this model, demonstrating that PML NBs are not accumulations of protein that are independent from the surrounding chromatin [4, 5].
Based on these results, we examined the fate of PML NBs when the cell undergoes DNA replication. Since PML NB integrity is dependent on chromatin contacts, we hypothesized that the bodies may be destabilized as these contacts are affected through changes in topological constraints during DNA replication. Indeed, the PML NBs do undergo fission and fusion events during the replication of euchromatin in early S-phase. More surprising, however, is that PML NB number increases by approximately two-fold during this time period. The strong connection between PML NBs and chromatin, and specifically euchromatin in early S-phase, leads to our hypothesis that PML NBs may interact with specific chromatin loci and contribute to the transcriptional regulation of these associated genes.

[1] Dellaire, G., Nisman, R., Bazett-Jones, D.P. (2004) Correlative Light and Electron Spectroscopic Imaging of Chromatin *in situ.* In Wu, C., Allis, C.D. (Ed). Methods in Enzymology 375, pp. 456-478.
[2] Ren, Y., Kruhlak, M.J., Bazett-Jones, D.P. (2003) Same serial section correlative light and energy filtered transmission electron microscopy. J. Histochemistry and Cytochemistry 51: 605-612.
[3] Dehghani, H., Dellaire, G., Bazett-Jones, D.P. (2005) Organization of chromatin in the interphase mammalian cell. Micron 36: 95-108.
[4] Eskiw, C.H., Dellaire, G., Bazett-Jones, D.P. (2004) Chromatin contributes to structural integrity of PML bodies through a SUMO-1-independent mechanism. Journal of Biological Chemistry 279: 9577-9585.
[5] Dellaire, G., Ching, R., Bazett-Jones, D.P. (2006) PML nuclear body number increases in early S-phase by a fission mechanism Journal of Cell Science 119:1026-33.

Single particle cryo-EM of the translational apparatus

Christian M.T. Spahn

Institut für Medizinische Physik und Biophysik, Charité - Universitätsmedizin Berlin, Ziegelstr. 5-9, 10117 Berlin, Germany

Cryo-electron microscopy (cryo-EM) in combination with digital image processing (single particle approach) is well suited to determine structures of large macromolecular complexes and machines at intermediate resolution (6-30 Å). In the course of image processing, tens to hundreds of thousands of such particle projections are combined to yield a 3-D density map. We are using this technique in order to obtain structural information about the translational apparatus. Ribosomal ligands can be directly visualized in cryo-EM maps and comparison of ribosomal complexes in different functional states can reveal large scale conformational changes within the translational machinery. Docking of atomic models for ribosomal components and ligands can lead to an interpretation in molecular terms.

Cryo-Electron Tomography of Neuronal Processes and Synapses

V. Lucic, T. Yang, W. Baumeister

Max Planck Institute for Biochemistry, Department of Structural Biology, Am Klopferspitz 18, D-82152 Martinsried, Germany

Cryo-electron tomography (cryo-ET) combines electron-tomography with the cryo-preparation that allows the investigation of frozen-hydrated samples free of artefacts caused by chemical fixation, dehydration, and uneven stain accumulation. It thus allows us to investigate three-dimensional structural features of biological material preserved in a "near-to-physiological" state.

We obtained tomograms of isolated glutamatergic synapses (from the synaptosomal fraction) under different experimental conditions. We have also optimized the cell culture conditions needed to obtain mature neuronal cultures from hippocampus suitable for cryo-ET, and obtained tomograms that show many known structural features of axonal and dendritic processes. These include cytoskeleton, postsynaptic density, and internal membranous compartments such as synaptic vesicles, transport vesicles, mitochondria, and smooth endoplasmic reticulum. This is the first time that these structures are observed in frozen-hydrated state within their native, cellular environment, in three dimensions.

We also improved our image processing methods in order to increase the usable resolution and analyze the structures present in our tomograms. To this end we optimized parameters in denoising algorithms developed for cryo-tomograms, and improved our parameter-free automated procedure for segmentation and morphological analysis of molecular complexes.

High Resolution Structured Illumination Microscopy

Rainer Heintzmann, Ondrej Mandula, Liisa Hirvonen, Yannick Colpin

King's College London, Randall Division, Guy's Campus, London SE1 1UL, United Kingdom

Key words: resolution, 4Pi, STED, structured illumination, patterned excitation

The concept of non-linear structured illumination is discussed along with other high resolution light microscopical approaches such as linear structured illumination and 4Pi microscopy.

In structured illumination the sample is illuminated with a number of different patterns of light. In our case this is a series of sinusoidal grids at different grid positions and orientations. Experimental datasets acquired under these conditions and reconstructed results from these data, demonstrating a resolution improvement of up to a factor of two over standard widefield microscopy are presented.

The non-linear approach of saturating optical transitions (for structured illumination as well as beam-scanning approaches) has a great potential especially in combination with photo-switchable dyes such as the recently released DRONPA protein by Atsushi Miyawaki's group.

Nanosizing and Structure Analysis of Gene Domains and Protein Complexes

U. J. Birk 1, D. Baddeley 1, H. Mathée 1, J. Reymann 1, C. Wotzlaw 2, C. Batram 1, Y. Weiland 1, J. Schwarz-Finsterle 1, C. Cardoso 3 and C. Cremer 1

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2 Institut for Physiology, University Essen, Germany
3 Max Delbrueck Center for Molecular Medicine, Robert Roessle Str. 50, 13125 Berlin, Germany

In order to extract information on gene activity “in vivo”, it would be highly advantageous to derive the degree of transcriptional activation directly from a (light-) microscopical visualization of structural changes e.g. of chromatin, formation of BioMolecular Machines and protein associations related to transcription and replication. Spatially Modulated Illumination (SMI) Microscopy provides a powerful tool to measure the extension of nuclear complexes such as specific gene domains[1], replication foci, transcription factories, etc. after fluorescence labeling, thus allowing the detection of differences in their conformational changes.
Recent advances of the SMI technique have facilitated a whole new range of applications e.g., measurements of the axial extensions of specific gene regions (e.g. p53 or cmyc) have been performed as a first step towards the microscopical detection of blood cancer related alterations correlated to the induction of translocations.[2] These measurements in principle allow the detection of structural changes of breakpoint regions in an early stage or closely related to the formation of the Philadelphia Chromosome or other translocations.
Moreover, in quantitative nuclear biology this “nanosizing” method allows a variety of applications. Examples are: gene expression analysis; gene copy analysis in 3D-intact nuclei; transcription factory analysis; protein cluster analysis. Here, we present studies of replication foci and of the c-myc gene region.
By the development of the Vertico-SMI with its upright orientation and its temperature controlled environment, considerable progress has been made in the direction of "in vivo" analyses. This is attested by first live cell acquisitions on membrane associated complexes (collaboration Prof. Gerisch). Additionally, the SMI measurements on replication foci have completed been by studies using the 4Pi microscope.

[1] Mathée, H., Baddeley, D., Wotzlaw, C., Fandrey, J., Cremer, C. and Birk, U. (2006). Histochem Cell Biol 125(1-2): 75-82.
[2] Hildenbrand, G., Rapp, A., Spoeri, U., Wagner, C., Cremer, C. and Hausmann, M. (2005). Biophys J 88(6): 4312-8.
[3] Wagner, C., Hildenbrand, G., Spöri, U. and Cremer, C. (2006). Optik 117(1-2): 1-48.

Ultra-high resolution light microscopy using structured illumination

L. Schermelleh 1, P. Carlton 2, J.W. Sedat 2 and H. Leonhardt 1

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2 University of California San Francisco (UCSF), Department of Biochemistry and Biophysics, USA

Studies of subcellular structures using state-of-the-art light microscopy have been restricted by the diffraction barrier of optical resolution that is 200-300 nm in the xy-plane and 500-800 nm along the z-axis. Recent advances have made possible to surpass the diffraction limit in axial direction using 4Pi microscopy [1] and additionally in lateral direction when combined with stimulated emission depletion (STED) microscopy [2]. An alternative approach developed at the UCSF uses the principle of structured illumination (SI) to improve lateral as well as axial resolution by a factor of two below the diffraction limit [3,4]. This technology has been implemented in a specially designed microscope platform, termed OMX, which provides unprecedented sensitivity and mechanical stability.
To explore the potential of the SI technology we have tested the OMX prototype on a wide variety of biological structures. We show here the conceptual basics of the OMX microscope and present exemplary high-resolution data on various structural features in mammalian cell nuclei. We show for the first time multi-fluorescence 3-dimensional (3D) data on the ultra-structural organization of the nuclear envelope and chromatin. In addition, we provide first light microscopical evidence for the organization of 300-800 nm sized DNA replication foci into smaller subunits of ~120 nm size. These results clearly demonstrate the potential of the OMX microscope for multi-wavelength 3D-imaging of biological samples with subdiffraction resolution that will allow new insights in biological structures and will help to narrow the gap between light and electron microscopy.

[1] Egner, A., and Hell, S.W. (2005). Trends Cell Biol. 15:207-15.
[2] Dyba, M., Jakobs S., and Hell, S.W. (2003). Nat Biotechnol. 21:1303-4.
[3] Gustafsson, M. G., Agard, D.A., Sedat, J.W. (1999) J Microsc. 195 (Pt 1): 10-16.
[4] Gustafsson, M. G. (2000) J Microsc. 198 (Pt 2): 82-7.

Dynamic Saturation Optical Microscopy

J. Sýkora 1, T. Dertinger 2, J. Enderlein 2

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2 Institute for Biological Information Processing 1, Forschungszentrum Jülich, D-52425 Jülich, Germany

A new scheme of fluorescence microscopy, Dynamic Optical Saturation Microscopy or DSOM[1], is presented allowing the breaking of the diffraction limit of optical microscopy by a factor of ca. four. It relies on fast temporal measurements of the fluorescence decay after sudden switch-on of the light excitation. The observed temporal dynamics of the fluorescence signal can be converted into information about the spatial distribution of fluorophores within the exciting laser focus. The proposed scheme is technically simple, allows resolution enhancement in three dimensions, and will be robust with respect to small optical aberrations as caused by refractive index variations in real samples.

[1] Enderlein, J. (2005) Appl. Phys. Lett. 87, 094105.

Fluorescent proteins: a toolkit from nature

Konstantin A. Lukyanov

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Micklukho-Maklaya 16/10, 117871, Moscow, Russia

Proteins of Green Fluorescent Protein (GFP) family represent a unique class of natural pigments that fully encoded in single genes. Their chromophores are formed from protein's amino acid residues without any external enzymes and cofactors except molecular oxygen. This feature makes GFP-like proteins an excellent tool for non-invasive fluorescent labeling of live cells and organisms. Since 1999, numerous GFP homologues have been discovered in Anthozoa, Hydrozoa and Copepoda species, showing a broad phylogenetic and spectral diversity of this protein family. Mutagenic studies gave rise to diversified and optimized variants of fluorescent proteins, which have never been encountered in nature. In particular, a number of so called photoactivatable fluorescent proteins were developed. Their fluorescence can be drastically increased by light of a specific wavelength and intensity enabling optical labeling and tracking of living cells, organelles and proteins. Recent develoment of first phototoxic fluorescent protein opens up new possibilities for precise light-induced cell killing and target protein inactivation. This talk gives an overview of the biochemical and spectral properties of GFP-like proteins and their most common applications for in vivo labeling.

New reporters for imaging protein trafficking and function in living cells

Alice Ting

MIT, 77 Massachusetts Avenue, Room 18-496, Cambridge, MA 02139, USA

Research in our lab focuses on the development of new methods, based in chemistry and biophysics, for studying protein trafficking and function in the live cell context. I will describe our efforts to harness the enzyme biotin ligase to label proteins site-specifically with a variety of biophysical probes in the cellular context. I will describe our application of this methodology for single-molecule imaging of glutamate receptors and synaptic adhesion proteins in live neurons using quantum dots. Other projects to be described include: new protein-protein interaction detection methodology, FRET reporters of enzyme activity, streptavidin engineering, and labeling with lipoic acid ligase.

Imaging of an enzyme-substrate complex in living cells

Carsten Schultz, Ivan Yudushkin, Andreas Schleifenbaum, Philippe Bastiaens

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We have developed a novel method for imaging enzyme-substrate (ES) intermediate in living cells by monitoring FRET between the donor fluorophore-tagged protein-tyrosine phosphatase PTP1B and a small synthetic acceptor-labeled substrate. The latter consists of the short receptor tyrosine kinase substrate peptide DAEDYL tagged with a rhodamine dye and equipped with a caged phosphate at the tyrosine residue. For passive entry into living cells, this compound was used as a membrane-permeant fusion to a penetratin sequence. Upon flash photolysis of the photoactivatable phosphate protecting group, we observed the establishment of a local steady-state flux of the reporter substrate through PTP1B due to a kinase/phosphatase reaction cycle. By analyzing a cellular map of the apparent K_M for PTP1B, reconstructed from images of the ES intermediate, we demonstrate that inside cells PTP1B exists as spatially separated kinetically distinct subpopulations, exhibiting high enzyme activity in the preinuclear region and low activity close to the plasma membrane.

Fluorescent Live Cell Imaging and Tracing
The challenge of long term live cell studies

Andrea Glatzel

Invitrogen, Technical Sales Specialist – Imaging & Microscopy

There are particular challenges associated with staining structures in living cells. The dyes used have to be permeable to the intact cell and possess some chemical characteristics that allow them to stain particular structures. The staining should be specific and the signals have to be stable for a long time without any photo bleaching. Fortunately there are a wide variety of dyes that possess such characteristics. This presentation will introduce some of these dyes, for example: Mitotrackers for mitochondria, Nucleic acid stains for nuclei, Lysotrackers and Lysosensors for acidic compartments, Lipophilic stains for membranes, ER Tracker for Endoplasmic reticulum and Ceramide conjugates for Golgi apparatus.
Furthermore, the Qdot® nanocrystals will be introduced. Qdot® nanocrystals are tiny semiconductor crystals (quantum dots) that emit brilliant light in colors from ultraviolet to infrared. Because of their strong photostability, Qdot® nanocrystals are extremely powerful tools for lice cell imaging. This presentation will focus on live cell applications, such as receptor internalization studies and live cell labeling.

Coumarinylmethyl esters for ultrafast release of high concentrations of cyclic nucleotides upon one- and two-photon photolysis

D. Lorenz, J. Eichhorst, B. Dekowski, V. Hagen and B. Wiesner

Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle Straße 10, 13125 Berlin, Germany

Caged compounds (biologically inert photoactivatable precursors) of cyclic nucleoside monophosphates (cNMPs) are powerful tools for studying spatiotemporal dynamics of cyclic nucleotide-dependent processes. Among these compounds, (coumarin-4-yl)methyl esters of cNMPs are most useful. They show no background bioactivity, are stable to solvolysis, and photolyze efficiently and extremely quickly.[1,2]
The combination of high solubility in aqueous buffer and high photosensitivity allows large and instantaneous concentration jumps of cNMPs upon flash photolysis both in solutions and inside living cells. Furthermore, we show that the new caged compounds photorelease the cNMPs upon one- and two-photon excitation. An additional advantage of these compounds is the possibility to determine quantitatively the amount of the photoreleased cyclic nucleotide inside cells using fluorescence measurements.
At least, the novel photoreleasable BCMACM protecting groups should be useful in caging and uncaging of other biomolecules with phosphate, carboxylate, carbonate or carbamate functionalities..

[1] Hagen, V., Benndorf, K., Kaupp, U.B. (2005) in *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules* (Eds: M. Goeldner and R. S. Givens), Wiley-VCH, Weinheim, pp. 155-178.
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Far-field fluorescence microscopy at the nanoscale: breaking Abbe's diffraction barrier by the RESOLFT concept

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Since its discovery by Abbe in 1873, the microscopy diffraction barrier has received a lot of attention. However, the ideas to improve the spatial resolution of a focusing (far-field) light microscope of the mid 20th century were not effective. Consequently, all far-field microscopes remained diffraction-limited in theory and practice. We discuss the breaking of the diffraction barrier in fluorescence microscopy through reversible saturable optical (fluorescence) transitions (RESOLFT). In a RESOLFT microscope, the diffraction barrier is broken by a saturated optical transition (depletion) between two states of a marker, whereby the transition is effected with an intensity distribution featuring one or more intensity minima (zero). The saturation level defines the size of the ultrasharp focal spot and/or the enlarged optical bandwidth (OTF). In a RESOLFT concept the resolution can be approximated by $\Delta x = \lambda/(\pi n \sqrt{I/I_{sat}})$, whereby I_{sat} is the characteristic intensity required for saturating the transition, and I denotes the intensity applied [1]. If the minima are produced with an aperture $n\sin\alpha$, we have $\Delta x \approx \lambda / 2n\sin\alpha \sqrt{1+ I/I_{sat}}$ which can be regarded as an extension of Abbe's equation [1, 2]. The diffraction-unlimited nature is reflected by the fact that Δx can be decreased by increasing $\zeta = I/I_{sat}$[1-7]. This is possible by increasing I or by lowering I_{sat} [1, 2, 8].
We show STED-microscopy displaying PSF of <20 nm FWHM, corresponding to a 15-fold enlargement of the OTF over Abbe's barrier [9]. The spot size reduction provided by STED also enables smaller probing spots in fluorescence fluctutation spectroscopy[10]. Finally, we report applications of STED-microscopy solving hitherto open biological questions of fundamental importance [11].

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Quantitative single pair FRET by pulsed interleaved excitation and fluorescence correlation spectroscopy

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With recent advances in sensitive fluorescence detection techniques single pair Förster resonant energy transfer (spFRET) is of increasing interest to detect e.g. co-localization of molecules or to measure conformational changes on the level of single molecules. However, in spite of the strong dependency of the energy transfer efficiency upon donor-acceptor separation quantitative results are barely to find, i.e. FRET experiments are interpreted qualitatively mainly.Quantitative analysis of spFRET is often hampered by a zero efficiency peak occurring in the FRET efficiency histogram, caused by molecules with missing or non fluorescent acceptor. Further problems arise from the presence of crosstalk due to imperfect spectral filtering, direct excitation of the acceptor as well as not directly measurable excitation- and quantum-efficiencies of the fluorophores and sensitivities of both detection channels.
To overcome the above-mentioned difficulties we applied[1] (dual color) pulsed interleaved excitation in FRET measurements (PIE-FRET) combined with time correlated single photon counting. Events contributing to the zero efficiency peak have been identified and eliminated to obtain clean FRET-histograms. Since direct acceptor excitation, molecular brightness of donor and acceptor fluorophores as well as crosstalk or leakage was determined by analyzing the same data-set with fluorescence correlation spectroscopy (FCS), all quantities required to analyze FRET-measurements properly were available. The advantages of the PIE-FRET approach were demonstrated with a poly-proline assay labeled with Alexa 555 as donor and Alexa 647 as acceptor, respectively.

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Combining spectroscopic methods for investigation of single autofluorescent proteins on the nanoscale

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Autofluorescent proteins are a field of great interest in various branches of biology and chemistry. Since they can serve for example as in vivo probes on the single-molecule scale a deeper understanding of the underlying photophysical processes is of great importance.

We present the combination of various optical techniques in the single-molecule regime to characterise spectroscopic properties of individual autofluorescent proteins, such as the bichromophoric DsRed mutant E8.
Using high resolution confocal microscopy investigation of structures on the nanoscale is accessible. In combination with high detection efficiency, ultra sensitive oberservation of individual protein entities is feasible.
Thus single molecule fluorescence studies give insights to the composition of tetrameric subunits[1] whereas surface enhanced Raman spectroscopy (SERS) allows for characterisation of binding properties of the proteins on metallic surfaces.[2]

Embedding the proteins between the mirrors of a microresonator can help to investigate fluorescence resonance energy transfer (FRET) within individual protein entities. Here we present a novel microcavity design based on a Farbry-Perot type interferometer[3] which allows for manipulation of both fluorescence and Raman emission of embedded autofluorescent proteins. Using time correlated single photon counting techniques FRET activity can be investigated due to changes in the radiative emission rate of the proteins.

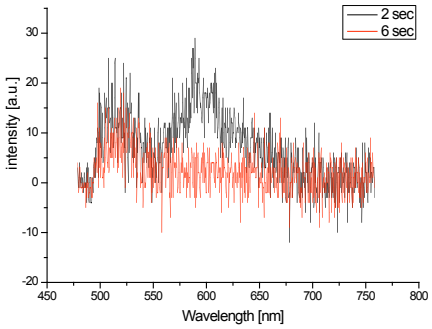

Fig 1: Single protein fluorescence spectrum showing selective photobleaching of one chromophore

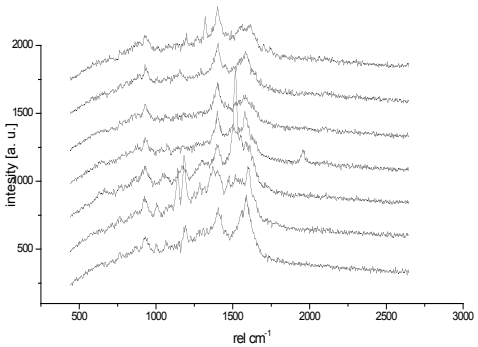

Fig 2: Series of Raman spectra of one single protein. High dynamics of single Raman lines are visible

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Alfred Nobel, and his Prizes

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When the organizers of this meeting invited me to present after-dinner remarks on an “entertaining subject”, I briefly considered the possibility that this meant a talk on my current research in the regular scientific sessions of the meeting would be less than entertaining. (Don’t answer that!) But then I quickly realized that I had been warmly offered a special privilege.

We are no strangers, in general, to the prizes that Alfred Nobel’s will set in motion, but there are intriguing elements of both his life and his prizes that are not well known. Aiming not to exceed the maximum 25 minutes that postprandial subjects can be expected to remain awake, I will outline some historical features of the Prizes and discuss current nomination and selection processes. We all have our favorite cases of perceived injustices, and I will describe a few of mine and offer speculations as to how they happened. I will close by trying to convey a little of the splendid Stockholm “Nobel Festival” proceedings themselves, as evocatively recorded by a 1950 Nobel laureate in an essay that is not widely known. The Nobel Award ceremonies, in all their complexity and grandeur, constitute the pinnacle of how institutional public relations (PR) can be done, this itself perhaps deserving of a “Nobel Prize in PR” (if Alfred had decreed one in his will).

Presuming that some of the audience is still awake when I finish we can toss my remarks around and share other perspectives- all to celebrate the joy of this profession of science we have been fortunate to choose, and whose spirits of shared purpose and upbeat camaraderie are surely among its most enabling elements, as this meeting has so richly demonstrated.

Four-Dimensional Imaging of Embryonic Tissue Dynamics

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The explosion of progress in the fields of cell biology, biochemistry, and molecular biology has offered unprecedented knowledge of the components involved in embryonic development. The dramatic progress of these reductionistic approaches poses the challenge of integrating this knowledge into an understanding of developmental mechanics that pattern and construct the embryo. The classic publications in the field of experimental embryology illustrate the power of describing cell behavior (cf. lineages, movements) and perturbing the embryo to test hypotheses of the underlying mechanisms. Advanced imaging techniques offer an important stepping-stone between these disparate approaches, permitting questions about cellular and molecular events to be posed in the most relevant setting of the intact embryo.
There are two major stumbling blocks. First, image acquisition by laser-scanning confocal microscopy is often too slow to capture individual image planes with sufficient rapidity. Second, it is difficult to capture 4-D data at sufficient temporal resolution especially if there is any motion. When the studied motion is periodic, such as for a beating heart, a way to circumvent this problem is to acquire, successively, sets of 2D+time data at increasing depths and later rearrange them to recover a 3D+time sequence. Recently we have utilized a newly-developed fast acquisition confocal microscope (Zeiss LSM 5 LIVE) to acquire very rapid, high-resolution 2D optical sections of fluorescently labeled cells in the developing zebrafish heart and have established image registration algorithms allowing for the registration of periodic movements. This method has allowed us to create 4-dimensional working models of the heart and analyze mechanical forces related to wall and valve motions from different stages of heart development. This approach is generalizable to many different setting in developmental biology and neurobiology.

Selective Plane Illumination Microscopy: Life sciences require the third dimension

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Novel technologies are required for three-dimensional cell biology and biophysics. By three-dimensional we refer to experimental conditions that essentially try to avoid hard and flat surfaces and favour unconstrained sample dynamics[1]. We believe that light-sheet-based microscopes are particularly well suited for studies of sensitive three-dimensional biological systems. The application of such instruments can be illustrated with examples from the biophysics of microtubule dynamics and three-dimensional cell cultures. Our experience leads us to suggest that three-dimensional approaches reveal new aspects of a system and enable experiments to be performed in a more physiological and hence clinically more relevant context.
A new implementation of the theta principle[2] takes advantage of parallel recording. This high-resolution light microscope[3] has been developed for the modern life sciences. It is designed to generate images of large samples (embryos, three-dimensional cell cultures) down to the sub-cellular level. The fundamental principle of EMBL's SPIM is the detection of fluorescence light perpendicular to the illumination axis. The illumination system provides the excitation light from the side of an object and hence selectively excites fluorophores within an entire plane. The illuminating light sheet overlaps with the focal plane of a detection system that consists of a long working distance lens and a CCD camera. SPIMaging provides optical sectioning directly. Photo bleaching outside the thin volume of interest is completely avoided. The photo toxicity of the illumination process is thus dramatically reduced. Since a SPIM performs well with long working distance lenses and has a good penetration depth millimetre-sized specimens can be observed in their entirety and as a function of time.
To further increase the resolution and the information content of the data stack, rotation of the sample adapts the excitation and detection axes to the sample. Parts of the sample that would otherwise be hidden or obscured become accessible. Data stacks recorded along different angles are combined in post-processing steps to yield high-resolution images of complete samples[4]. The 3D resolution is then dominated by the lateral resolution and resolution becomes identical, i.e. isotropic along all directions.
Since the SPIMaging process provides for an excellent signal to noise ratio an image processing procedure such as deconvolution works extremely well and is easily integrated into image data processing steps. For the past years various different species (Medaka, drosophila, yeast,…) were observed with the instrument.

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Tracking of stem cell behavior at the single cell level: new tools for old questions

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Despite intensive research, many long-standing questions of hematopoietic stem cell biology remain unsolved. One major reason is the fact that hematopoiesis is usually followed by analyzing the fate of populations of cells – not of individual cells - at very few time points of an experiment, and without knowing (or quickly loosing) their individual identities. The static picture yielded by this approach makes it impossible to appreciate the dynamic developmental processes leading to the (re)generation of the blood system from single hematopoietic stem cells (HSCs). Real-time tracking of individual cells in culture, tissues or whole organisms would be an extremely powerful approach to fully understand the developmental complexity of hematopoiesis. However, many of the needed tools are still under development and their application in stem cell research remains difficult. Here, a computer aided incubation and bioimaging system was developed to follow the fate of individual cells over long periods of time (up to several weeks) with highest temporal resolution. New software modules for data acquisition and analysis were written, helping to control microscopes and record, display and statistically analyze the divisional history, position, properties, lineage choice etc. of all individual cells derived from HSCs. In combination with newly generated transgenic mice expressing different sub-cellular forms of fluorescent proteins in specific hematopoietic lineages, this system is used to analyze the embryonal generation of blood cells and the behavior of individual adult HSC and all of their progeny over many generations at the single cell level.

Quantitative analysis of Plasmodium sporozoites motility

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Plasmodium sporozoites form in parasitic cysts at the gut wall of an infested Anopheles mosquito. After release from these cysts, they invade the salivary glands from where they are transmitted into the skin of the host during the bite of a mosquito. Sporozoites move slowly within salivary glands but speed up considerably once injected into the skin, where they move to invade blood and lymph vessels [1-3]. We aim to understand the role of motility during transmission and the biophyiscal basis of sporozoite movement. To gain insights into transmission we image Plasmodium sporozoites that express the green fluorescent protein in their cytoplasm within living mice after natural transmission. Quantitative analysis shows how sporozoites modulate their speed and move with distinct patterns in different environments. For a better understanding of the mechanical forces applied by the parasite during motility to its substrate we use traction force microscopy. For this, sporozoites are isolated from the salivary glands of mosquitoes and imaged on flexible sheets of polyacrylamide gels imbedded with fluorescent beads [4].

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Following the Synthesis and Travels of Single mRNAs in Living Cells

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We developed a system for analyzing gene expression in real-time, and used it to follow individual mRNPs. An array of genes coding for a functional mRNA that contains 24 repeats of the MS2 coat protein binding motif combined with the MS2 coat protein fused to GFP or YFP allowed us to analyze the kinetics of transcription in real time and to detect single molecules of RNA in live cells [1] [2]. In these studies we used photo-bleaching and photo-activation of GFP-labeled mRNAs and of a GFP-polII fusion protein. The analysis demonstrated that nuclear RNA synthesis was governed by complex kinetics and that subsequent RNA mobility was not directed, but governed by rules of simple diffusion.
Once in the cytoplasm, mRNAs diffuse but also can be directed to their destinations [3]. Static mRNAs may be in the process of translating proteins [4]. Beta-actin mRNA can be localized to sites of active F-actin polymerization in migrating fibroblasts or growing neurons. In yeast, mating-type switching depends on the asymmetric distribution of Ash1 protein, due to localized translation of *ASH1* mRNA sorted to the bud tip. In both of these systems, it is possible to observe this process directly in real time in order to deduce the mechanisms governing localization. This demonstrates that a spatial and temporal sequential process regulates RNA localization. The RNA binding protein, ZBP1 contributes to the asymmetric sorting of beta-actin mRNA by inhibiting its translation until it reaches its destination [5]. Disruption of this process severely affects the extension of neurites in both neuronal cell lines and primary hippocampal cells. Translation can be verified using a novel method to tag nascent polypeptides. Like beta-actin mRNA, translation of *ASH1* mRNA must also be inhibited before it is localized. One of the proteins that binds this mRNA is She2p [6] and another is Puf6p [7], a novel protein of the PUF family, originally identified as a translational inhibitor in Drosophila and C. elegans. By use of the variety of technologies developed, we can envision following an RNA molecule from its birth through its travels and finally to its expression.

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Studying membrane turnover and complex formation of vesicular coat proteins in living cells

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An experimental strategy using live cell imaging, fluorescence recovery after photobleaching (FRAP) and mathematical modeling of the experimental data to study molecular aspect of vesicular membrane turnover and assembly at exit sites of the endoplasmice reticulum (ERES) will be presented. The data obtained with this approach show that the presence of transport competent secretory cargo and the interaction of the Sec23/24p COPII sub-complex with the dynactin complex component $p150^{Glued}$ stabilize COPII at ERES. This prevents premature COPII disassembly and provides the time to enable cargo sorting, concentration and subsequent carrier formation. These data are in support of a model by which membranes of the early secretory pathway can be linked to motors and microtubules for subsequent organization and movement to the Golgi apparatus.

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Centromere assembly through highly immobile and some mobile foundation kinetochore proteins

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We have analysed the dynamic behaviour of the foundation kinetochore proteins CENP-A, CENP-B, CENP-C, CENP-I, and hMis12 in living human cells using fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS). In interphase cells, CENP-A, CENP-B, CENP-C, and CENP-I are stable components of the kinetochore over hours. In contrast, hMis12 rapidly and completely exchanges within seconds. During mitosis CENP-A, CENP-C, and CENP-I remain stably associated with kinetochores, while CENP-B becomes mobile and, strikingly, hMis12 becomes completely immobilized at kinetochores. FCS detected soluble pools with diffusion coefficients between 1 and 2 $\mu m^2 sec^{-1}$. This diffusional behaviour is consistent with transient chromatin interactions of foundation kinetochore proteins outside of centromeric DNA. In contrast to many other subnuclear complexes but similar to nucleosomes, our observations identify the kinetochore as a nuclear DNA/multiprotein assembly which is not determined by a constant flux of exchanging components. However, alterations in the mobility of specific kinetochore proteins appear to be associated with the changing functional properties of centromeres during mitosis.

The 3-dimensional organization of free-running actin waves

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The actin system within a cell is capable of forming organized complexes several micrometer in size through the recruitment of proteins from the cytosol. We have analysed the self-organization of actin into travelling waves that can apply forces on the membrane, pushing it forward when they move against the cell border. Basis of the wave formation is the spatio-temporal control of actin polymerisation and depolymerization. Proteins constituting the waves include a motor protein, myosin-IB, the Arp2/3 complex responsible for the nucleation of dendritic structures of actin filaments, as well as coronin, an inhibitor of the Arp213 complex.
By TIRF microscopy and spinning disc confocal imaging we have determined the organization of actin waves in parallel and normal to the plasma membrane. Myosin-IB forms the front of the waves and is closely linked to the membrane. Arp2/3 is distributed throughout the entire actin filament network. Coronin is recruited in a characteristic pattern, forming an oblique layer extending the waves from a position most distant from the plasma membrane at the front of the wave to a position close to the membrane at the back of the wave. The positioning of actin-associated proteins in the waves reflects their functions in a programmed cycle of actin polymerisation and depolymerization.

Analysis of XMAP215-mediated microtubule growth by TIRF microscopy

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The proper regulation of microtubule dynamics is essential for many cellular processes. One enzyme that plays a critical role in modulating microtubule behavior is the conserved protein XMAP215, a potent microtubule stabilizer that functions by increasing the rate of microtubule growth and nucleation. We have developed an *in vitro* assay using total-internal-reflection fluorescence microscopy (TIRF) to investigate the interactions of XMAP215 with both stabilized and dynamic microtubules. We demonstrate with single molecule resolution that XMAP215-GFP diffuses in 1D along the microtubule lattice of stabilized microtubules. We further show that XMAP215 recruits free tubulin from solution and that this XMAP215/tubulin complex also diffuses along the microtubule. These results suggest a model whereby XMAP215 promotes microtubule growth by shuttling tubulin dimers to the plus end of microtubules.
To examine this hypothesis further, we modified our TIRF assay to allow for the visualization of dynamic microtubule growth. We demonstrate that the diffusion of XMAP215 is important for microtubule nucleation and growth. Furthermore, we are able to monitor the localization of single XMAP215-GFP molecules along growing microtubules. Utilizing these approaches, we are currently dissecting the mechanistic role of XMAP215 in regulating microtubule dynamics.

Replication fork progression in the absence of processive DNA synthesis

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The precise coordination of the different steps of DNA replication is critical for the maintenance of the genome stability. Much of the current knowledge of the eukaryotic DNA replication machinery has been gained from biochemical studies in vitro. However, very little is known about the assembly and coordination of its different enzymatic activities in vivo. Inhibition of DNA replication with aphidicolin is widely used to analyze the different steps of replication. Aphidicolin specifically inhibits DNA polymerase α [1] and DNA polymerase δ [2] by competing with dNTPs for binding to the polymerase.
By inhibition of the DNA polymerases with aphidicolin, we have probed the mechanisms coupling the various parts of the replication machinery and their response to polymerase stalling in living mammalian cells. A combination of in situ extractions with live cell microscopy including dual colour time-lapse, dual colour FRAP and photoactivation combined with FRAP was employed to analyze the dynamic properties of proteins involved in DNA replication. We observed an accumulation of proteins involved in the initiation process, a transient arrest of DNA polymerase δ and disassembly of elongation proteins at replication foci. These results indicated an uncoupling of the initiation from the processive DNA elongation machinery and a concomitant increase of ssDNA. Within the DNA elongation machinery itself, the Okazaki fragment maturation proteins disassembled before the polymerase clamp PCNA which itself was followed by polymerase δ as determined by live cell microscopy. This demonstrated the flexible and dynamic response of the replication machinery to challenges during DNA replication.

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Visualisation of interactions in living cells using FLIM and FRET

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In order to understand functional changes within living neurons (e.g. during synaptogenesis), a precise investigation of the molecular interactions between macromolecules within their natural environment is a prerequisite. Previous investigations for studying the dynamics within living cells were based on the use of video or confocal laser scanning microscopy in combination with vital fluorescent markers. Unfortunately, most of these visualisation techniques (fluorescence/CLSM/2-Photon, etc) use high illumination intensities that cause considerable photo-dynamic reactions within living cells and, therefore, do not allow a non-distorted continuous observation of dynamic processes for a longer period. We were successful in creating a microscopic set up with ultra sensitive detectors like DL (Delay Line) and QA (Quadrant Anode) for studying interactions in living neurons by a combination of FLIM and FRET.
A ratiometric chloride indicator, Clomeleon was used initially to study FRET in living neurons. In Clomeleon, a chloride sensitive variant of YFP called Topaz was linked with a relatively chloride insensitive CFP by using a 24 amino acid linker. We studied the fluorescence dynamics of Clomeleon in hippocampal cell cultures at three different stages of maturation (DIV 7, 10 and 15). In young hippocampal cells (DIV 7), we found a quenching of the YFP moiety of Clomeleon in the majority of neurons, indicating a reduction or absence of energy transfer which might be due to the high intracellular Cl^- concentration present. In the majority of mature cells, quenching of YFP seemed highly reduced, due to better energy transfer from CFP in presence of lower Cl^- concentration. Analysis of the Decay Associated Spectra (DAS) corroborated the presence of an energy transfer with a negative pre-exponential factor corresponding to the lifetimes participating in the energy transfer.
Bassoon is among the earliest proteins to be incorporated into nascent synapses, consistent with its role in active zone assembly. Despite their exquisite localization in the CAZ (cytomatix at the active zone) and putative role in active zone assembly, little is known about the mechanisms of CAZ assembly and of targeting of these cytomatrix proteins to active zones. Previous studies have shown that these proteins play a pivotal role as scaffolding constituents within the presynaptic boutons of developing synaptic contacts. In order to address this question, we investigated xFP-fusion constructs of the large neuron-specific protein (Bassoon: 420 kDa) and its putative binding partners identified so far including the transcriptional corepressor CtBP1 and Cast1, a member of the ERC family of proteins. We were able to study the interactions of these proteins in heterologous cell lines as well as in dissociated hippocampal cell cultures. A multi-exponential decay analysis was done and a reduction in the average lifetime of the donor was observed in presence of energy transfer. With the current study, we hope to get a better understanding of the different mechanisms underlying targeting of these proteins and synapse assembly.

Single molecule biochemistry in living cells

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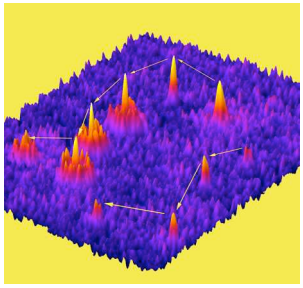

Single Molecule Tracking (SMT) via high-speed fluorescence microscopy has become a powerful tool for accessing directly the mobility of single molecules in solution. Recently we were able to demonstrate its capability as a complementary method to Fluorescence Correlation Spectroscopy (FSC) to study fast moving small proteins.[1] So far just a few studies have exploited the potential of SMT to measure diffusion of molecules inside the cellular environment. The distribution of pre-mRNA processing splicing factors is an important example of the fascinating spatio-temporal organization of mammalian cell nuclei. We examined the dynamics of U1 small nuclear ribonucleoprotein particles (U1snRNPs) inside and outside the “nuclear speckles” of living HeLa cells, and tracked single U1snRNPs in cell nuclei at a spatial precision of approximately 30 nm and a time resolution of 5 ms. Strikingly we observed numerous binding events, extending over hundreds of milliseconds at very specific sites, which were not restricted to the speckles. Detailed data analysis revealed different fractions of U1snRNPs, namely those presumably active in splicing, those contained in very large supramolecular complexes performing slow tumbling motions, and fast mobile ones. Next we examined the movement of an average-sized inert tracer protein (streptavidin, 60 kDa) inside living cell nuclei. The mobility inside nucleoli and pericentric heterochromatin domains was compared to the mobility within the remaining nucleoplasm. The results indicated that all nuclear subcompartments were easily and similar accessible for small proteins like streptavidin, even the molecular crowded and densely packed pericentric heterochromatin. Pericentric heterochromatin tended to transiently trap probe molecules more than nucleoplasm and nucleoli. Trapping was minimal and mobility was highest inside the nucleoli. Our data show that light-microscopy imaging, nanolocalization and tracking of single molecules in living cells allows detailed insight into intranuclear pathways and the “elucidation” of bimolecular interactions.

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Functional Studies of the Mitotic Spindle in Cells and Tissues

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The assembly of mitotic chromosomes and kinetochores is critical for proper mitotic chromosome segregation. We have combined proteomics and timelapse imaging to identify new mitotic chromosome proteins using. Xenopus cell-free extracts are well-established for the mechanistic study of the cell cycle. In addition, in vitro-assembled chromatin can be easily purified from Xenopus extracts, enabling a proteomic analysis of chromatin at defined cell cycle states using 2D gel analysis and LC-MSMS. In this primary screen, we identified >450 proteins specifically associated with in vitro assembled mitotic chromosomes, ~60 of which had no described function, 6 of which were selected for further analysis based on homology with other organisms and the presence of identifiable sequence domains. These “hits” were characterized using high-resolution timelapse imaging and RNAi. One of these, Bod1, is a kinetochore component that shows significant sequence similarity to Dam1p, a critical yeast kinetochore component. Bod1 siRNA in HeLa cells causes a strong defect in mitotic chromosome biorientation. Timelapse imaging of HeLa cells expressing GFP-CENP-B and mCherry1-tubulin shows that mitotic centromeres in Bod1-depleted cells still oscillate and generate force at microtubule ends. Bod1-depleted cells have intact spindle checkpoints, correctly localise Aurora B protein kinase, but fail to correctly localise the Aurora B substrate MCAK, a critical regulator of microtubule dynamics, at the centromere of mono-oriented mitotic chromosomes. Our data suggest that Bod1 is a critical biorientation determinant that functions downstream of Aurora B anndd helps target MCAK to the centromere.

We have also studied mitosis within the neural tube of the chick embryo. We visualise cells electroporated with GFP-tubulin transiting the cell cycle and making developmental choices for up to 40 hours in the chick embryonic neural tube. We observe that neurons are generated in two ways in the spinal cord; by neural stem cell divisions and by cells dividing to produce two terminally differentiating neurons. Of the 23 complete lineages we have been able to monitor, 18 were progenitor generating divisions of which 5/18 (30%) divided with a parallel cleavage plane classically characteristic of stem cell divisions. Of the 5 neuron generating divisions, two cells divided with perpendicular cleavages to give two neurons each and only one of three neural stem cell divisions divided with a parallel cleavage plane. Thus, cleavage plane orientation does not predict cell fate outcome in this tissue. Our data also suggest that cell fate may be partially controlled by cell cycle time. All progenitor generating divisions were shorter (<18h 45min) than neural stem cell divisions, suggesting that a longer cell cycle is a prerequisite for neuron production. To test this, we followed 30 lineages in slices treated with FGF, which represses neuronal differentiation. All FGF lineages contained only progenitor generating divisions and no instances of neuronal behavior were observed. Our ultimate goal is to combine these two approaches to understand how mitosis drives fate choices in living tissue.

How to deal with photobleaching and phototoxicity in life-cell imaging

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Photobleaching and phototoxicity are two of the most important limitations of fluorescence live-cell microscopy. Photobleaching and phototoxicity are caused by several photochemical processes after excitation of fluorophores in the sample. The most straightforward way to reduce photobleaching and phototoxicity in fluorescence microscopy is by reducing the dose of light for the excitation of fluorophores. However, reduction of excitation light will lead to loss of image quality (reduced signal to noise ratio (S/N) and/or reduced spatial and temporal resolution).

Controlled Light Exposure Microscopy (CLEM) reduces phototoxicity and photobleaching without loosing image quality. This technology is based on a non-uniform illumination of the fluorescent sample that allows tuning the light dose for every individual pixel. We have implemented CLEM in a confocal microscope. Results show that CLEM reduce photobleaching by a factor of 7 in tobacco plant cells expressing GFP-MAP4. In HeLa cells expressing chromatin associated H2B-GFP the production of reactive oxygen species (ROS) is reduced 8-fold causing a 6 times longer scanning time without noticeable cell damage.

In this presentation we introduce the general problem of photobleaching and phototoxicity and we will show how CLEM can help to reduce it.

Fully integrated light and atomic force microscopy to study cellular structure and adhesion

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The atomic force microscope (AFM) is a mechanical microscope, where contrast during imaging is generated depending on the structural properties of a sample. When imaging cells, the heterogeneous nature of the cell surface means that it can be difficult to interpret the significance of structural data from the atomic force images alone. The combination of AFM with light microscopy extends the potential for this imaging technique. To be truly effective however, not only must the AFM be integrated into an inverted light microscope, but the imaging software must be able to accurately correlate a field imaged using the AFM with an optical image acquired from the light microscope. New software for the JPK Nanowizard™ has been developed that can use tip location to map an optical image into the AFM image space, made possible by the fact that the instrument uses closed loop positioning of the AFM tip. This means that, tip position for imaging, force-spectroscopy measurements and manipulation, can now be chosen with respect to the integrated optical image.

Time-Resolved Confocal Fluorescence Microscopy with Single Molecule Sensitivity: Novel Technical Features and Applications for FLIM, FRET and FCS

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In recent years time-resolved fluorescence measurement and analysis techniques became a standard in single molecule microscopy. However, considering the equipment and experimental implementation they are typically still an add on and offer only limited possibilities to study the mutual dependencies with common intensity and spectral information. In contrast, we are using a specially designed instrument ("MicroTime 200") with an unrestricted photon data acquisition approach which allows to store spatial, temporal, spectral and intensity information in a generalized format preserving the full experimental information. This data format allows us not only to easily study dependencies between various fluorescence parameters but also to use, for example, the photon arrival time for sorting and weighting the detected photons to improve the significance in common FCS and single molecule FRET analysis schemes.
The power of this approach will be demonstrated for different techniques: In FCS experiments the concentration determination accuracy can be easily improved by a simple time-gated photon analysis to suppress the fast decaying background signal. A more detailed analysis of the arrival times allows even to separate FCS curves for species which differ in their fluorescence lifetime but, for example, cannot be distinguished spectrally[1,2]. In multichromophoric systems like a photonic wire which undergoes unidirectional multistep FRET the lifetime information complements significantly the intensity based analysis and helps to assign the respective FRET partners. Moreover, together with pulsed excitation the time-correlated analysis enables directly to take advantage of alternating multi-colour laser excitation. This pulsed interleaved excitation (PIE) can be used to identify and rule out inactive FRET molecules that cause interfering artefacts in standard FRET efficiency analysis [3].
We used a piezo scanner based confocal microscope equipped with compact picosecond pulsed diode lasers as excitation sources. The timing perfomance could be significantly increased by using new SPAD detectors from MPD that enable, in conjunction with the PicoHarp TCSPC electronics, an overall IRF of less than 120 ps maintaining single molecule sensitivity. The PicoHarp allows also to record arrival times up to minutes with ps-accuracy enabling the determination of fluorescence correlation curves from ps to minutes (total correlation) just with one single device.

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Single molecule microscopy using focal plane illumination

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Single molecule fluorescence microscopy performed in spatially extended samples suffers from the drawbacks of a low axial resolution, high background light and rapid photo-bleaching[1]. To overcome these disadvantages we use a focal plane illumination instead of the standard epi-illumination. We create a 20x20µm wide and 2µm thick light sheet within the focal plane by a custom made cylindrical lens system (NA = 0.33). In this manner an optical sectioning microscope is created [2,3]. The light sheet is produced inside a water chamber, where the sample is fixed within an agarose gel cylinder [2]. Fluorescence light is detected perpendicular to the illumination plane by a water-dipping microscope objective lens (60X, NA = 1.1) and imaged onto a CCD camera. With this setup, only the plane of interest is illuminated and therefore affected by photo-bleaching. Excitation of the fluorescence light in the focal plane only leads to a background reduction. The axial resolution is determined by the light sheet thickness and the resolving power of the detection objective lens. The penetration depth of the optical sectioning is limited by the working distance of the water-dipping microscope objective (w.d. = 2.5mm). With this setup we achieve an axial resolution of approximately 830nm and a significant reduction of fluorescence background in single molecule imaging in extended 3-dimensional systems such as cell nuclei.

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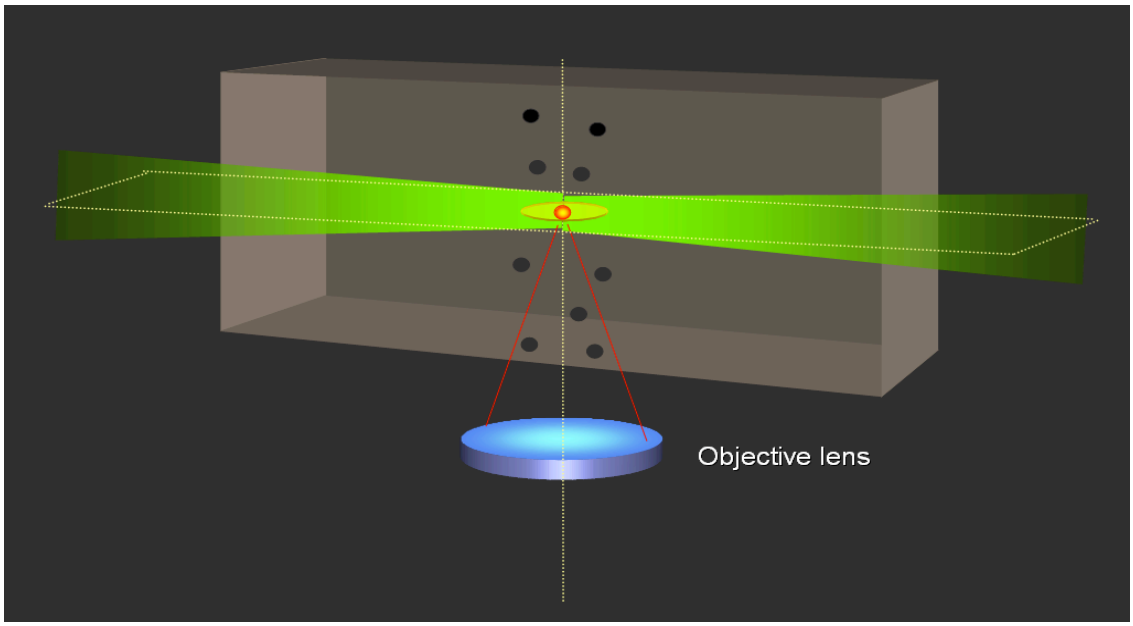

Schematic view of the focal plane and perpendicular detection of the fluorescence light

Using photobleaching microscopy to dissect spliceosome dynamics in the nucleus

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The spliceosome is a very dynamic RNA-protein macromolecular machine that is responsible for the splicing of intronic sequences from pre-mRNA, a crucial step in the pathway of mRNA biogenesis of higher eukaryotes. The spliceosome undergoes major structural changes during the splicing reaction and its components must be recycled for a new round of splicing after exon ligation and release of mRNA. Although the spliceosome cycle has been extensively studied at the molecular level, very little is known about the dynamics of spliceosome components in vivo.
We are using FRAP (Fluorescence Recovery After Photobleaching) and FLIP (Fluorescence Loss Induced by Photobleaching) to analyze the mobility and kinetic behavior of spliceosome components in the nucleus of living HeLa cells. In addition we perform Acceptor Photobleaching FRET to visualize and spatially map the interactions between the different splicing factors. Taken together, our results reveal that spliceosome components are highly dynamic in the nucleus and that splicing factors assemble into pre-spliceosomal complexes that accumulate in dedicated nuclear compartments.

Tools for the analysis of cellular and intra-cellular activity at high spatial and temporal resolution

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Recent tools developed for i) multi-modal image acquisition combining multi-dimensional image acquisition together with laser assisted techniques (“fast” 4-5D deconvolution microscopy, FRAP/PA techniques), and ii) quantification of static and dynamic data of microscopic images (segmentation, object tracking, colocalization, …) will be presented. The talk will be focused on distinct biological applications underlining the benefit of these approaches, providing an overview these approaches dedicated to the understanding of fast and complex subcellular activities.

Building high resolution maps of gene positions in yeast nuclei by automated 3D image analysis

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The localization of specific genes inside the nuclear volume is both non random and linked to transcriptional activity[1]. Intranuclear localization thus provides an important gene regulation mechanism, but its generality and the underlying physical mechanisms are still poorly understood. To explore this further, we need more systematic and detailed quantitative descriptions of 3D gene locations with respect to important spatial landmarks such as the nuclear envelope, the nucleolus and the spindle pole body. Because genes are also known to undergo stochastic motions[2,3], a statistical analysis on large amounts of data is essential. Here, we will report on our efforts to build high resolution maps of spatial gene distributions by automated analysis of thousands of 3D images of fluorescently labeled genes in yeast nuclei. Yeast allows easy manipulation of the transcriptional status, but the cell diameter of only 2-3 micrometers means that images of intranuclear structures are strongly blurred by diffraction. Nevertheless, we will show that an image processing approach based on statistical detection and estimation can locate genes relative to other nuclear structures with a precision higher than the diffraction limit. Geometric alignement of the locations extracted from each nucleus then enable us to condense the information from whole populations into meaningful probability density maps (see example below). Such maps provide a visualization of where genes are located relative to the nucleolus and other nuclear landmarks. We expect that this approach, which allows a significantly deeper look into the nuclear architecture, will be of great value to the study of gene positioning and function.

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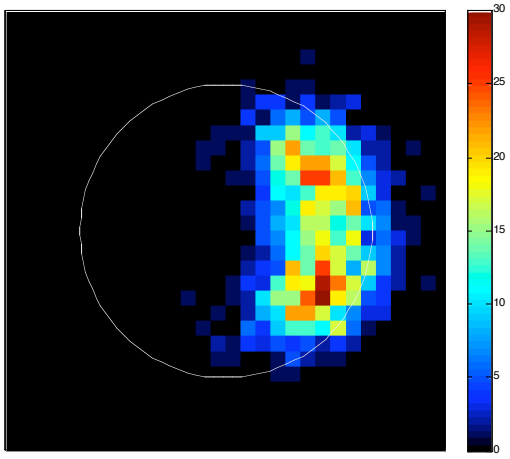

IMAGING OF COLLAGEN IN LIVE TISSUES, USING A NEW FLUORESCENT PROBE PhF

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Background. Collagen forms an intricate meshwork of fibrils (diameter of 10-300 nm) and fibres (diameter of several micrometers) that ensure the mechanical strength of skin, tendons, cartilage, bone and connective tissue. Many genetic and acquired disorders arise from improper collagen polymerisation, structure, fibre formation and age-related collagen denaturation. Non-invasive three-dimensional fluorescence imaging of collagenous structures in living tissues would be beneficial in studies of collagen in health and disease. So far no specific vital fluorescent label for collagen has been known. Thus, currently the best established method of high resolution imaging of collagenous structures in live tissues is second harmonic generation (SHG) microscopy. Autofluorescence and reflected light imaging of collagen present alternatives but these methods are not specific to collagen, therefore their usefulness in studies of collagen in live cells and tissues is limited. **Goal.** The goal of this work was to establish a method of labelling collagen fibrils and fibres with a new fluorescent probe PhF, demonstrate the usefulness of this labelling technique in confocal microscopy of live cells and tissues, and decipher the molecular mechanism of binding of PhF to polymerised collagen. **Results.** We describe a new, simple and rapid method of high resolution imaging of collagen fibrils and fibres in live tissues. It employs a newly synthesized, low molecular weight fluorescent probe (PhF), which exhibits high affinity to collagen and penetrates tissue readily. Specificity of binding of PhF to collagen type I polymerised in vitro was confirmed by simultaneous fluorescence and reflected light imaging. Confocal imaging of live tissues demonstrated that various collagenous structures, including thin reticular fibres in heart and blood vessel walls, collagen fibres in tendon, diaphragm and skeletal muscle as well as other tissues were labelled specifically. The mechanism of binding of PhF to collagen was studied by microscopy and molecular modelling. FRAP studies demonstrated that PhF binds to collagen noncovalently. Molecular modelling suggested that PhF molecule assumes a curved shape, which fits into the groove on a triple-helix of a collagen. The fluorescent probe appears to bind to a surface of a collagen molecule by hydrophobic forces and two hydrogen bridges. **Conclusion.** PhF labels collagen in live tissues readily. Thus, it may be useful in live tissue confocal studies of resistance to mechanical stress, fibril formation, building of collagen fibre networks, rearrangement of fibres, and structural differences between collagenous networks in healthy and diseased tissues.

Mathematical modeling of cellular reaction networks with special focus on nucleotide excision repair

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I will start with an overview on recent results on modelling cellular reaction networks including metabolic networks and signal transduction networks [1-3]. Then I will present a new model of nucleotide excision repair (NER) which requires the concerted action of many different proteins that assemble at sites of damaged DNA in a sequential fashion. The model is based on data for the assembly kinetics of the putative damage-recognition factor XPC-HR23B at sites of DNA damage in the nuclei of living cells. Combined with other in vivo kinetic data we scrutinized the dynamic behaviour of the nucleotide excision repair process in detail. A sequential assembly mechanism appears remarkably advantageous in terms of repair efficiency. Alternative mechanisms for repairosome formation, including random assembly and preassembly, can readily become kinetically unfavourable. Our work provides a kinetic framework for NER and rationalizes why many multiprotein processes within the cell nucleus show sequential assembly strategy.

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Dynamics of Transcriptional Regulation in Living Cells

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Transcription is now recognised as an inherently dynamic process. The functional contribution of dynamic events to regulating transcription, however, is largely unknown. We have therefore sought to elucidate whether polymerase assembly dynamics contribute to regulation of transcription in vivo. Using RNA polymerase I as a model system, we have used a combination of live cell imaging and computational analysis to study how the assembly dynamics of RNA polymerase I change in response to up-regulation of transcription during the cell cycle. We have measured the kinetics of RNA Pol I pre-initiation factors, assembly factors and polymerase subunits under conditions of differential transcriptional output. During S-phase, when RNA Pol I activity is maximal, polymerase subunits and assembly factors are retained for an increased amount of time in the nucleolus compared to G1, when rRNA transcription is low. Importantly, the kinetics of the non-elongating pre-initiation factor UBF remain unchanged between the two conditions. Application of quantitative computational analyses shows that the residence time of assembly factors and polymerase subunits at the promoter is increased when transcription is up-regulated. The consequences of these residence time changes include an increased probability that these proteins interact with the promoter and a corresponding increase in the probability that individual proteins are assembled into an elongating polymerase. To test this hypothesis, we measured the effect on polymerase dynamics of mutating a MAPK phosphorylation site in the assembly factor TIF-IA. This interaction occurs at the TIF-IA – polymerase interaction interface and has been shown to decrease pre-rRNA transcription levels. The presence of this mutant does not affect the kinetics of the pre-initiation or assembly factors, crucially however, the recovery kinetics of the polymerase subunits are increased. Most importantly, the recovery of the different polymerase subunits is perturbed to differing extents suggesting independent roles in the assembly process. Computational analysis shows that the mutation does not affect the probability that a polymerase subunit will bind to the promoter but significantly decreases the probability that the subunit will be incorporated into an elongating polymerase. We therefore propose that differences in the dynamics of RNA Pol I assembly lead to differential transcriptional output, through an increase in the efficiency of polymerase assembly. Our results suggest that changes in the dynamic behavior of RNA Pol I subunits contribute to transcriptional regulation and our observations point to a dynamic interplay between transcriptional activity and protein interactions.

Analyzing the transcriptional kinetics of an active gene in living cells

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The kinetic properties of the reactions leading to gene expression within the genetic environment of a living cell are not well understood. A live-cell system was used for following real-time transcription dynamics on a particular gene[1,2]. Fluorescent reporters for RNA polymerase II, a specific DNA locus and its synthesized mRNA were utilized. By applying photobleaching and photoactivation techniques combined with mechanistic mathematical modeling, the kinetics of transcription from this gene were resolved. We obtained an *in vivo* measurement for the elongation speed of RNA polymerase II and identified pausing as an unexpectedly significant process occurring during RNA polymerase II elongation. Polymerase pre-initiation and initiation processes were quantified and found to be mainly abortive processes limiting mRNA production. This approach provides a mechanistic *in vivo* description of RNA polymerase II gene-specific transcriptional kinetics and provides new clues to gene regulation and current models of transcription.

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Dynamic simulation of active and inactive chromatin domains

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Presently, a rosette like structure of chromatin loops is assumed to exist for the experimentally observed chromosomal foci. On the basis of the 30nm fiber a polymer model was implemented which allows the dynamic folding in rosette like structures when attractive sites were distributed along the fiber.
These attractive sites, representing condensing agents (HMG/SAR, HP1, cohesin, condensin, DNA-DNA interaction etc.), tend to aggregate and form rosettes. The diameter of these rosettes depends on the number of attractive sites and therefore also on the loop size. A crucial question now is the accessibility of these rosettes for transcription factor complexes. Presently there still two opinions about the aggregation process of transcription and splicing factors. They might form functional complexes directly at the sites of genes. Alternatively, (sub-) complexes are built up at distant sites, in the so called inter-chromatin regions, and subsequently reach the genes by passive diffusion. In the later case, such aggregates are in the order between 55 and 70nm in diameter and have to access the active genes to allow transcription. Brownian Dynamics simulations of rosettes/TF complexes were performed to investigate the dynamics of the interaction between the 30nm fibre and the protein complexes.

P1: **Spatially Modulated Illumination microscopy as a tool for the measurement of nuclear protein complexes**

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Spatially Modulated Illumination (SMI) microscopy is a method of structured illumination microscopy allowing very precise measurements of parameters such as the size and position of fluorescently labelled biological structures 1,2. This technique uses the interference of two plane waves within the sample volume to create an illumination structure in the axial direction. The degree to which this illumination structure is conserved in the resulting image depends on the size of the object. A quantification of this 'modulation' in the images allows size estimates to be obtained for objects with sizes in the range between ~40 and 200nm.

We have applied this technique to the determination of replication foci sizes and found these to be in the order of 120nm, with a large spread and no significant dependence on the position in S-Phase. These results confirm those found in the an electron microscopy study performed by Koberna et Al 3, but offer a much larger sample size yielding better statistics and, through the labelling of both replicated DNA (BrdU incorporation) and PCNA, show that the size of the machinery and the replicated DNA is approximately the same.

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P2: **Influence of different cell fixation procedures on the localization and extent of the centromeric region of chromosome 8 in human fibroblasts**

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To evalute quantitatively the influence of different preparations / fixation protocols on the three dimensional organization of a specific chromatin region, the extension and positioning of a centromer 8 probe was determined in normal fibroblast nuclei. The shape of counterstained interphase nuclei was evaluated in 3D with a Confocal Laser Scanning Microscope applying three different cell fixation protocols: (1) non-hypotonic methanol/acetic acid (3:1), (2) hypotonic methanol/acetic acid (3:1), potassium chloride treated (75mM) for a metaphase spread, and (3) formaldehyde (4%). For the analysis we used VH7 cells, normal human embrionic foreskin fibroblasts. The centromeric region of chromosome 8 was labelled by conventional Fluorescence In Situ Hybridization (FISH) applying a commercial probe of Qbiogene. The evaluations of the shape of the nucleus, the localization and the extent of the chromosome region were made by specific image analysis algorithms. The extensions of the centromeric region of chromosome 8 were determined in the two lateral directions. It could be shown, that the fixation of cells by methanol/acetic acid leads to an increase of the nuclear volume, a decrease of the axial extent, and a flattening of the nuclei. The volume of VH7 nuclei after the hybridization procedure is reduced in comparison to non treated nuclei from the same suspension. Despite the strong influence of the fixation on the nuclear shape, our data suggest that the fixation procedure influences the localization of the centromeric region of chromosome 8 not very much. Also the extent of this chromosomal structure is not highly changed by hybridization. The comparison of the extents of centromere 8 between metaphase and interphase samples revealed lower differences as expected.

P3: **BioAFM - A NanoImaging tool for the combination with advanced optical methods**

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JPK Instruments enables NanoImaging for the Life Science community by introducing Atomic Force Microscopy (AFM) to users with biological applications. The BioAFM allows nm resolution imaging on native objects, force measurements on a single molecule scale and nano manipulation of biological objects. The JPK NanoWizard combines these capabilities with all imaging methods provided by current optical microscopy. Therefore a large variety of research fields such as cell biology, biomaterials, molecular biology and bionics can benefit from the synergy of both technologies.

P4: **Time-resolved fluorescence correlation spectroscopy combined with lifetime tuning: new perspectives in supported phospholipid bilayers' research**

A. Benda 1, V. Faguĺová 1, A.G. Deineka 2, J. Enderlein 3 and M. Hof 1

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Recently, the measurement capabilities of FCS have been enriched by using pulsed lasers as well as time-tagged time-resolved data storage. Similar to two-color FCS, a time-resolved fluorescence correlation spectroscopy[1] (TR-FCS) experiment enables to calculate simultaneously the auto- and cross- correlation functions of fluorescent species with different excited state lifetimes[2]. While in two-color FCS the chromophores have to exhibit large differences in their spectral characteristics, sound TR-FCS experiments can be straight forward performed if the fluorescence lifetimes differ by a factor of 2. Since the fluorescence lifetimes of chromophores are sensitive to changes in their particular microenvironment, TR-FCS offers the unique advantage to characterize two different diffusion processes using single dye labeling. In this work we exploit the modification of lifetimes of labeled lipids in SPBs by a presence of the solid support. Light absorbing supports shorten the lifetime of a dye, which is located in a nanometer distance to the support, to a value which is significantly shorter than a lifetime of the same dye located in freely diffusing small unilamellar vesicles (SUVs). To this end, we firstly characterize how the lifetime of labeled lipids in SPBs can be tuned by varying the solid support as well as the dye-support distance. Secondly, we show that TR-FCS enables to characterize simultaneously 2-dimensional lipid diffusion in SPBs and 3-dimensional vesicle diffusion in a single experiment. Diffusion coefficients obtained by this new approach are compared to diffusion coefficients obtained by standard FCS on isolated SPBs.

[1] Böhmer, M., Wahl, M., Rahn, H. J., Erdmann, R., Enderlein, J. (2002) Chemical Physics Letters, 353, (5-6), 439-445.
[2] Benda, A., Hof, M., Wahl, M., Patting, M., Erdmann, R., Kapusta, P. (2005) Review of Scientific Instruments**,** 76, (3), Art. No. 033106.

P5: **Microfluidic approaches for the study of cell dynamics**

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The sensing of chemical signals by cells and the chemically triggered movement of cells along gradients of chemoattracting agents is central to a large number of biological and medical problems, including morphogenesis, immune response, and cancer metastasis. The recent development of novel microfluidic techniques allows the tailoring of devices, where flow conditions and distributions of chemical species can be precisely controlled and manipulated on the length scales of individual cells. In combination with modern fluorescent microscopy imaging techniques, signaling and cell movement can be systematically investigated in a well-defined environment. As an example, we show the application of a microfluidic gradient generator to study migration and intracellular protein dynamics during chemotaxis of the social ameba *Dictyostelium discoideum*, one of the most prominent model organisms for the study of chemotactic behavior.

P6: **Local Protein/Gene Density Measurements Using Structured Illumination**

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Spatially Modulated Illumination (SMI) Microscopy[1] in its present form provides a reliable, fast and robust tool to determine object parameters far below the resolution limit of conventional (e.g. confocal) light microscopes. Even without directly increasing the optical resolution, precise information about the objects' axial extensions[2] at their (relative) positions[3] can be determined by nanometer controlled scanning either of the object or of the phase of the interference pattern. In this report we applied SMI and 4Pi microscopy to determine changes of the local refractive index at discrete cites within the cell nucleus. These changes may be related to local accumulations of nucleic acids and proteins, having a higher refractive index than the surrounding media. To extract the data based on the local refractive index, we have used fluorescence markers targeted to specific cites within the cell nucleus. Different fluorescence labeling techniques are applicable, such as gene sequence targeting by fluorescence in situ hybridization (FISH), antibody labeling of proteins, or the use of membrane specific dyes. By selecting appropriate targets (we used e.g. polymerase II enzymes) the whole nucleus can be scanned. In SMI microscopy, the grid of discrete points for which the refractive index and thus the protein concentration can be extracted is limited by the diffraction barrier. Two data points have to be separated by at least the full-width-at-half-maximum of the microscope detection point spread function.
We found a variation of the local refractive index at labeled polymerase II cites of about 4% throughout the nucleus. This variability is not correlated to the accumulation and the extension of the polymerase II complexes, which have been determined in a previous experiment.[4] The variations could be related to other transcriptional active proteins, but could also be due to different compactions of the DNA fiber.
Altogether, we present a method to precisely obtain a map of the local refractive index inside of cell nuclei, which provides another contrasting mechanism for visualizing sub cellular structures. Being based on fluorescence microscopy, this technique can yield true 3D information on the local refractive index, which can then enter reconstruction algorithms such as those used in 3D deconvolution, providing a way to quantitatively predict changes of the microscope PSF.

[1] H. Mathée, D. Baddeley, C. Wotzlaw, J. Fandrey, C. Cremer and U. Birk (2006). Histochem Cell Biol 125(1-2): 75-82.
[2] G. Hildenbrand, A. Rapp, U. Spoeri, C. Wagner, C. Cremer and M. Hausmann (2005). Biophys J 88(6): 4312-8.
[3] C. Wagner, G. Hildenbrand, U. Spöri and C. Cremer (2006). Optik 117(1-2): 1-48.

P7: **Nanosizing and Structure Analysis of Gene Domains and Protein Complexes**

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In order to extract information on gene activity “in vivo”, it would be highly advantageous to derive the degree of transcriptional activation directly from a (light-) microscopical visualization of structural changes e.g. of chromatin, formation of BioMolecular Machines and protein associations related to transcription and replication. Spatially Modulated Illumination (SMI) Microscopy provides a powerful tool to measure the extension of nuclear complexes such as specific gene domains[1], replication foci, transcription factories, etc. after fluorescence labeling, thus allowing the detection of differences in their conformational changes.
Recent advances of the SMI technique have facilitated a whole new range of applications e.g., measurements of the axial extensions of specific gene regions (e.g. p53 or cmyc) have been performed as a first step towards the microscopical detection of blood cancer related alterations correlated to the induction of translocations.[2] These measurements in principle allow the detection of structural changes of breakpoint regions in an early stage or closely related to the formation of the Philadelphia Chromosome or other translocations.
Moreover, in quantitative nuclear biology this “nanosizing” method allows a variety of applications. Examples are: gene expression analysis; gene copy analysis in 3D-intact nuclei; transcription factory analysis; protein cluster analysis. Here, we present studies of replication foci and of the c-myc gene region.
By the development of the Vertico-SMI with its upright orientation and its temperature controlled environment, considerable progress has been made in the direction of "in vivo" analyses. This is attested by first live cell acquisitions on membrane associated complexes (collaboration Prof. Gerisch). Additionally, the SMI measurements on replication foci have completed been by studies using the 4Pi microscope.

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[2] Hildenbrand, G., Rapp, A., Spoeri, U., Wagner, C., Cremer, C. and Hausmann, M. (2005). Biophys J 88(6): 4312-8.
[3] Wagner, C., Hildenbrand, G., Spöri, U. and Cremer, C. (2006). Optik 117(1-2): 1-48.

P8: **Probing Nuclear Environments by Single Particle Tracking (SPT)**

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Single Particle Tracking (SPT) via high-speed fluorescence microscopy has become a powerful tool for directly accessing the mobility of molecules within biological membranes. However, only few studies have exploited the potential of SPT for measuring diffusion of molecules in other cellular compartments. Additionally, due to its high spatial resolution, SPT is an ideal method for studying the movements of diffusing single proteins within a cellular environment. We introduced a high sensitivity high speed CCD camera into a widefield microscope setup, which allowed us to probe the movements of an average-sized nonfunctional protein (streptavidin) in the nucleus of living cells. Streptavidin was labeled with the fluorophore Cy5, coupled with a nuclear localization signal and microinjected into the cytoplasm of mouse myoblast cells. The nuclear subcompartments nucleolus and pericentric heterochromatin are large enough to allow studying the mobility of streptavidin inside these compartments. We labeled the pericentric heterochromatin by transfecting the cells with a GFP fusion of MeCP2, a protein that binds pericentric satellite DNA. Our results indicate that nuclear subcompartments are easily accessible for small proteins as streptavidin (60 kDa in its tetrameric form), but nevertheless differences exist concerning the concentration in the individual compartments as well as a varying tendency for getting trapped within nuclear subcompartments on the time scale of a few milliseconds.

P9: **Time-Resolved Confocal Fluorescence Microscopy with Single Molecule Sensitivity: Novel Technical Features and Applications for FLIM, FRET and FCS**

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In recent years time-resolved fluorescence measurement and analysis techniques became a standard in single molecule microscopy. However, considering the equipment and experimental implementation they are typically still an add on and offer only limited possibilities to study the mutual dependencies with common intensity and spectral information. In contrast, we are using a specially designed instrument ("MicroTime 200") with an unrestricted photon data acquisition approach which allows to store spatial, temporal, spectral and intensity information in a generalized format preserving the full experimental information. This data format allows us not only to easily study dependencies between various fluorescence parameters but also to use, for example, the photon arrival time for sorting and weighting the detected photons to improve the significance in common FCS and single molecule FRET analysis schemes.
The power of this approach will be demonstrated for different techniques: In FCS experiments the concentration determination accuracy can be easily improved by a simple time-gated photon analysis to suppress the fast decaying background signal. A more detailed analysis of the arrival times allows even to separate FCS curves for species which differ in their fluorescence lifetime but, for example, cannot be distinguished spectrally[1,2]. In multichromophoric systems like a photonic wire which undergoes unidirectional multistep FRET the lifetime information complements significantly the intensity based analysis and helps to assign the respective FRET partners. Moreover, together with pulsed excitation the time-correlated analysis enables directly to take advantage of alternating multi-colour laser excitation. This pulsed interleaved excitation (PIE) can be used to identify and rule out inactive FRET molecules that cause interfering artefacts in standard FRET efficiency analysis [3].
We used a piezo scanner based confocal microscope equipped with compact picosecond pulsed diode lasers as excitation sources. The timing perfomance could be significantly increased by using new SPAD detectors from MPD that enable, in conjunction with the PicoHarp TCSPC electronics, an overall IRF of less than 120 ps maintaining single molecule sensitivity. The PicoHarp allows also to record arrival times up to minutes with ps-accuracy enabling the determination of fluorescence correlation curves from ps to minutes (total correlation) just with one single device.

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P10: **Dynamic anatomy of replicating chromatin domains**

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The process of DNA replication is a complex spatiotemporal process, which involves all levels of hierarchical chromatin (un)packing. To be replicated chromatin must be decondensed but the scale of such decondensation is yet to be investigated. It is also largely unknown how replication gets propagated along the chromosomes and whether both processes are interconnected. Existing experimental evidence can be interpreted within the context of two paradigms. The first one implies assembly of replication factories at the surface of large scale chromatin domains, decondensation of the chromatin and its translocation from inside of the domain to the replication machinery and back, followed by recondensation [1, 2]. The second paradigm implies assembly of the replication machinery at initial chromatin sites with subsequent propagation of the replication 'wave' by preferential de novo assembly of the replication machinery at the neighboring chromosomal regions likely involving local changes in chromatin condensation [3].
To study the dynamic organization of chromatin replication and its progression in mammalian cells we used multidimensional time-lapse CLSM of living mouse cells. To analyze the position of replication machinery labeled with GFP-PCNA relative to adjacent chromatin domains in vivo, minor and major satellite heterochromatin domains were visualized using CENPB-DsRed and MECP2–YFP proteins. Our study has shown that replication of heterochromatin domains in mammalian cells occurs via sequential local decondensation of chromatin and penetration of replication machinery inside macro chromatin structures. The replication of mouse pericentric heterochromatin is directional and minor repeat regions of individual chromosomes replicate after major satellite repeats. A model for the replication of heterochromatin will be discussed.

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P11: **Protein translocation through the nuclear pore complex in living cells: analyzed one molecule at a time**

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All protein and RNP traffic between cell nucleus and cytoplasm occurs via the nuclear pore complex (NPC), which is located within the nuclear envelope of eukaryotic cells. NPCs are extremely large (~60 MDa) supramolecular protein complexes with a barrel-shaped geometry with a diameter of 125 nm and a length of approximately 120 nm [1]. Proteins and RNP complexes with molecular weights greater than 40 kDa are translocated by the NPC in a signal-dependent manner by import factors, e.g., karyopherin α, karyopherin β or karyopherin β2.
In continuation of our work on digitonin-permeabilized cells[2] we used fluorescence microscopy to study nucleocytoplasmic transport at the single-molecule level in living cells. To this end the fluorescently labeled transport receptors kapα2, kapβ1, kapβ1 ΔN44, kapβ2 and a model transport substrate, NLS-BSA-Alexa488 were microinjected into the cytoplasm of living HeLa cells expressing GFP-Pom121. The translocation process of single molecules could be studied at a time resolution of 5 ms with a localization precision of ≈ 35 nm. The distribution of the respective binding sites was determined in relation to the position of GFP-POM121, which was used as an internal reference. Furthermore, the interaction times of the proteins with the NPCs were analyzed. The dissociation kinetics from the NPCs could well be described by a monoexponential decay, and average interaction durations between 4 and 10 ms were determined. Comparison of these values to those determined in digitonin-permeabilized cells – which are only slightly larger – suggests that the ongoing export occurring in living cells does not significantly modify the translocation duration of a given protein. Together with the known bulk transport rates[3] our data suggest, that nucleocytoplasmic transport occurs via multiple parallel pathways within single NPCs.

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P12: **Quantitative measurments of preservation of large-scale chromatin structure after different fixation procedures used for FISH experiments**

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Fluorescence in situ hybridization (FISH) has been widely used to investigate the organization of chromatin in the cell nucleus. Two fixation procedures were typically applied, (i) buffered formaldehyde and (ii) hypotonic shock with methanol acetic acid (MAA) fixation followed by dropping of nuclei on glass slides and air drying. Here, we compared the effects of these two procedures on nuclear morphology and on FISH signals by confocal microscopy and quantitative image analysis. We investigated mouse erythroleukemia (MEL) and mouse embryonic stem cells because their clusters of subcentromeric heterochromatin provide an easy means to assess preservation of chromatin. Qualitative and quantitative analysis revealed that formaldehyde fixation provided good preservation of large-scale chromatin structures while classical MAA fixation following hypotonic treatment severely impaired nuclear shape and led to disruption of chromosome territories, heterochromatin structures and large transgene arrays. Our data show that such preparations do not faithfully reflect in vivo nuclear architecture.

P13: **The nuclear envelope is not a diffusion barrier for calcium in hippocampal neurons**

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The ability to increase the intracellular calcium concentration following synaptic activity is a fundamental feature of neurons and controls many neuronal processes. Calcium is required for the regulation of gene expression that affects synaptic connectivity, promotes survival, or causes cell death [1]. For synapse-to-nucleus communication, neurons exploit the spatial and temporal diversity of calcium transients associated with electrical activation [2]. In the nucleus, calcium signals induced by neural firing patterns are integrated to specify the transcriptional output [3,4]. However, calcium signalling in the cell nucleus is still discussed controversially 5. The questions concern primarily the calcium permeability of the nuclear pore complex, the existence of nuclear calcium stores, and the role of calcium release channels (i.e. Ins(1,4,5)P3 and ryanodine receptors) and calcium up-take system in the generation and clearance of nuclear calcium transients [6,7]. To study calcium dynamics in the cytosol and the nucleus, we established laser-assisted photolysis of caged calcium compounds, which enables us to generate spatially distinct calcium signals in hippocampal neurons. We show that changes in the cytosolic calcium concentration lead to rapid changes in the nuclear calcium concentration and vice versa. Calcium appears to diffuse freely between the cytosol and the nucleus and equilibrates quickly between the two compartments. The nuclear envelope does not attenuate or slow down calcium flux into the nucleus and thus does not represent a diffusion barrier for calcium.

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P14: **Dynamic Saturation Optical Microscopy**

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A new scheme of fluorescence microscopy, Dynamic Optical Saturation Microscopy or DSOM[1], is presented allowing the breaking of the diffraction limit of optical microscopy by a factor of ca. four. It relies on fast temporal measurements of the fluorescence decay after sudden switch-on of the light excitation. The observed temporal dynamics of the fluorescence signal can be converted into information about the spatial distribution of fluorophores within the exciting laser focus. The proposed scheme is technically simple, allows resolution enhancement in three dimensions, and will be robust with respect to small optical aberrations as caused by refractive index variations in real samples.

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P15: **Assembly of influenza virus proteins in lipid-rafts analyzed with FRET**

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Influenza virus is an enveloped, negative-stranded RNA virus. Assembly of influenza virus requires enrichment of its glycoproteins hemagglutinin (HA) and neuraminidase (NA) and of the peripheral matrixprotein M1 at the plasma membrane of infected cells. Enrichment is supposed to occur in lipid-rafts, cholesterol- and sphigomyelin-rich subdomains, which include only a few cellular components, such as palmitoylated and glypidated proteins. HA and NA have intrinsic signals for raft-localization, wheras M1 localizes to rafts only when the viral glycoproteins are present. Oligomerization of M1 is believed to merge small HA-containing rafts to larger domains. Most of these results were obtained with the Triton-extraction method and it is controversial whether insolubility in detergent reflects assocciation of proteins with rafts inside cells.
Our recently started project aims at verifying this model using fluorescence-resonance-energy transfer (FRET) between viral proteins and markers for rafts inside cells. We established cells expressing the 20 N-terminal amino acids of GAP-43 including two palmitoylation sites fused to the yellow-fluorescent-protein (YFP-Mem). Confocal fluorescence micoroscopy and total internal reflection fluorescence microscopy (TIRFM) showed that the construct localizes to the inner leaflet of the plasma membrane. YFP-Mem is incorporated into budding viruses suggesting a close association with viral components. Extraction of cells with Triton suggests that YFP-Mem is present in detergent-resistant membranes. Clustering of YFP-Mem will be confirmed with a homo-FRET (anisotropy) approach. Having established YFP-Mem as a raft-marker, we will use FRET to analyze whether HA fused to CFP interacts with YFP-Mem. The effect of M1-coexpression on raft-clustering will then be investigated.

P16: **Dimerization of corticotropin-releasing factor receptor type 1: FRET measurements to analyzing the dimer-monomer ratio**

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As described previously receptor dimerization of G-protein-coupled receptors may influence signaling, trafficking and regulation in vivo. Up to now, most studies aiming at the possible role of receptor dimerization in receptor activation and signal transduction are focused on class A GPCRs. In the present study the dimerization behavior of the corticotropin-releasing factor receptor type 1 (CRF_1R), which belongs to class B of GPCRs and plays an important role in coordination of the immune response, stress and learning behavior, was investigated using fluorescence resonance energy transfer (FRET). For this purpose we generated fusion proteins of CRF_1R tagged to their C-terminus to a cyan or yellow fluorescent protein, which can be used as a FRET pair. Binding studies verified that the receptor constructs were able to bind their natural ligands in a manner comparable to the wild type receptor, while cAMP accumulation proved the functionality of the constructs. For the excitation of the CFP we have use the two-photon technique, since thereby almost no direct YFP excitation is possible.
In microscopic studies, a dimerization of the CRF_1R was observed, but the addition of either CRF-related agonists or antagonists did not show any dose-related increase of the observed FRET, indicating that the dimer-monomer ratio is not changed upon their addition. Further we have tried to calculate the relationship between monomers and dimers.

P17: **Gold nanorods as non bleaching labels: a novel confocal microscopy technique to detect and characterize their scattering patterns**

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A novel method for imaging of single metallic nanoparticles is presented. This method provides information about the particle shape, size and orientation. Using radially and azimuthally polarized doughnut modes [1, 2] for excitation confocal images of single gold nanoparticles i.e Au nanospheres and Au nanorods immobilized on glass are collected. Both the scattered light from the single particles and the reflected light from the glass slide were detected using the same objective, and contribute to the image. We study experimentally and theoretically the pattern rendered from gold nanospheres and nonorods. It was found that these patterns reflect the different particle polarizability and are sensitive to index of refraction mismatch of the interface placed in focus. In case of nanorods, the scattering patterns reveal the orientation of the particles as shown in figure 1. and also permit to determine the distance between two rods with a precision of a few nanometers. This novel technique provides a promising tool for the visualization of nonbleaching labels in bio sciences.

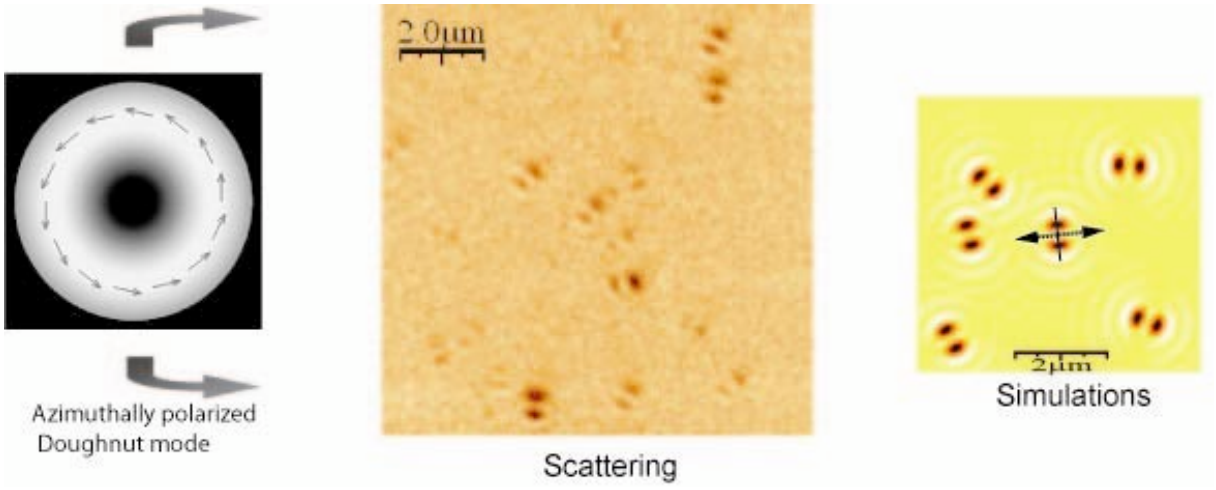

Fig. 1: Left, Schematic representation of an azimuthally polarized doughnut mode; centre, scattering image of gold nanorods using for excitation a azimuthally polarized doughnut mode; right, simulations of scattering patterns of gold nanorods.

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P18: **The 3-dimensional organization of free-running actin waves**

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The actin system within a cell is capable of forming organized complexes several micrometer in size through the recruitment of proteins from the cytosol. We have analysed the self-organization of actin into travelling waves that can apply forces on the membrane, pushing it forward when they move against the cell border. Basis of the wave formation is the spatio-temporal control of actin polymerisation and depolymerization. Proteins constituting the waves include a motor protein, myosin-IB, the Arp2/3 complex responsible for the nucleation of dendritic structures of actin filaments, as well as coronin, an inhibitor of the Arp213 complex.
By TIRF microscopy and spinning disc confocal imaging we have determined the organization of actin waves in parallel and normal to the plasma membrane. Myosin-IB forms the front of the waves and is closely linked to the membrane. Arp2/3 is distributed throughout the entire actin filament network. Coronin is recruited in a characteristic pattern, forming an oblique layer extending the waves from a position most distant from the plasma membrane at the front of the wave to a position close to the membrane at the back of the wave. The positioning of actin-associated proteins in the waves reflects their functions in a programmed cycle of actin polymerisation and depolymerization.

P19: **Fluorescent Live Cell Imaging and Tracing**
The challenge of long term live cell studies

Andrea Glatzel

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There are particular challenges associated with staining structures in living cells. The dyes used have to be permeable to the intact cell and possess some chemical characteristics that allow them to stain particular structures. The staining should be specific and the signals have to be stable for a long time without any photo bleaching. Fortunately there are a wide variety of dyes that possess such characteristics. This presentation will introduce some of these dyes, for example: Mitotrackers for mitochondria, Nucleic acid stains for nuclei, Lysotrackers and Lysosensors for acidic compartments, Lipophilic stains for membranes, ER Tracker for Endoplasmic reticulum and Ceramide conjugates for Golgi apparatus.
Furthermore, the Qdot® nanocrystals will be introduced. Qdot® nanocrystals are tiny semiconductor crystals (quantum dots) that emit brilliant light in colors from ultraviolet to infrared. Because of their strong photostability, Qdot® nanocrystals are extremely powerful tools for lice cell imaging. This presentation will focus on live cell applications, such as receptor internalization studies and live cell labeling.

P20: **Replication fork progression in the absence of processive DNA synthesis**

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The precise coordination of the different steps of DNA replication is critical for the maintenance of the genome stability. Much of the current knowledge of the eukaryotic DNA replication machinery has been gained from biochemical studies in vitro. However, very little is known about the assembly and coordination of its different enzymatic activities in vivo. Inhibition of DNA replication with aphidicolin is widely used to analyze the different steps of replication. Aphidicolin specifically inhibits DNA polymerase α [1] and DNA polymerase δ [2] by competing with dNTPs for binding to the polymerase.
By inhibition of the DNA polymerases with aphidicolin, we have probed the mechanisms coupling the various parts of the replication machinery and their response to polymerase stalling in living mammalian cells. A combination of in situ extractions with live cell microscopy including dual colour time-lapse, dual colour FRAP and photoactivation combined with FRAP was employed to analyze the dynamic properties of proteins involved in DNA replication. We observed an accumulation of proteins involved in the initiation process, a transient arrest of DNA polymerase δ and disassembly of elongation proteins at replication foci. These results indicated an uncoupling of the initiation from the processive DNA elongation machinery and a concomitant increase of ssDNA. Within the DNA elongation machinery itself, the Okazaki fragment maturation proteins disassembled before the polymerase clamp PCNA which itself was followed by polymerase δ as determined by live cell microscopy. This demonstrated the flexible and dynamic response of the replication machinery to challenges during DNA replication.

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P21: **Live cell imaging of Dictyostelium amoebae elucidates functions of DdLIS1 and DdDCX, two Microtubule-associated Proteins involved in Lissencephaly**

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Lissencephaly results from impaired cell migration of neuronal precursors during development of the neocortex. Mutations in the genes encoding LIS1 and doublecortin (DCX) are responsible for approximately 80% of all cases of lissencephaly. Since Dictyostelium is an outstanding model for studies on cell migration and development, we analyzed the homologues of LIS1 and doublecortin.
Dictyostelium LIS1 (DdLIS1) is a microtubule (MT) and centrosome-associated protein. Co-precipitation experiments revealed that DdLIS1 interacts with dynein, DdCP224 and the small GTPase Rac1A. Replacement of the DdLIS1 gene by the hypomorphic D327H allele had no strong effects on development but disrupted various dynein-associated functions and altered actin dynamics. Our results show that DdLIS1 is required for maintenance of the MT cytoskeleton, Golgi apparatus and nucleus/centrosome association, and they suggest that LIS1-dependent alterations of actin dynamics could also contribute to defects in neuronal migration in lissencephaly patients.
DCX is characterized by two conserved tandem repeats of the DC-domain that are involved in microtubule (MT) binding. Although DCX has only been described in vertebrates where its expression was restricted to migrating, developing neurons, we have found a homologue in Dictyostelium. Its sequence similarity is mainly restricted to the DC-domains. GFP-DdDCX was localized along MTs and at the cell cortex where it colocalizes with actin. The latter can be attributed to a F-actin binding and bundling activity of DdDCX. Endogenous DdDCX was distributed similarly but it was only detectable after 8 h of development. DdDCX null mutants exhibited no phenotype. However, DdDCX null mutants additionally carrying the hypomorphic DdLIS1 allele are defective in formation of streams and aggregates during development, although they show normal chemotaxis along external cAMP signals. Stream formation turned out to be independent of MTs. Taken together, this suggests that DdDCX and DdLIS1 cooperate in cAMP signaling and that both proteins may also be involved in cytoskeleton-independent functions in brain development.

P22: **Single molecule biochemistry in living cells**

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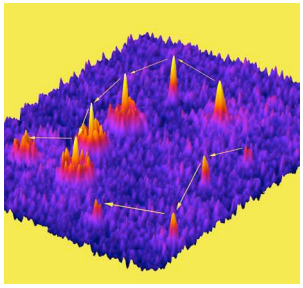

Single Molecule Tracking (SMT) via high-speed fluorescence microscopy has become a powerful tool for accessing directly the mobility of single molecules in solution. Recently we were able to demonstrate its capability as a complementary method to Fluorescence Correlation Spectroscopy (FSC) to study fast moving small proteins.[1] So far just a few studies have exploited the potential of SMT to measure diffusion of molecules inside the cellular environment. The distribution of pre-mRNA processing splicing factors is an important example of the fascinating spatio-temporal organization of mammalian cell nuclei. We examined the dynamics of U1 small nuclear ribonucleoprotein particles (U1snRNPs) inside and outside the “nuclear speckles” of living HeLa cells, and tracked single U1snRNPs in cell nuclei at a spatial precision of approximately 30 nm and a time resolution of 5 ms. Strikingly we observed numerous binding events, extending over hundreds of milliseconds at very specific sites, which were not restricted to the speckles. Detailed data analysis revealed different fractions of U1snRNPs, namely those presumably active in splicing, those contained in very large supramolecular complexes performing slow tumbling motions, and fast mobile ones. Next we examined the movement of an average-sized inert tracer protein (streptavidin, 60 kDa) inside living cell nuclei. The mobility inside nucleoli and pericentric heterochromatin domains was compared to the mobility within the remaining nucleoplasm. The results indicated that all nuclear subcompartments were easily and similar accessible for small proteins like streptavidin, even the molecular crowded and densely packed pericentric heterochromatin. Pericentric heterochromatin tended to transiently trap probe molecules more than nucleoplasm and nucleoli. Trapping was minimal and mobility was highest inside the nucleoli. Our data show that light-microscopy imaging, nanolocalization and tracking of single molecules in living cells allows detailed insight into intranuclear pathways and the “elucidation” of bimolecular interactions.

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P23: **Employing z-scan method in lateral diffusion measurements by means of fluorescence correlation spectroscopy: model systems versus cellular applications**

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It was shown that for the determination of lateral diffusion coefficients by the means of fluorescence correlation spectroscopy (FCS), in contrast to three dimensional systems, the spatial profile of the laser beam has to be taken into account. Benda[1] et al developed the Z-scan method based on moving the plane of interest along the z-axis of the beam. Fitting the z-position dependences of FCS read-out parameters (i.e. average number of particles in the illuminated area and average time the fluorophor needs to diffuse through the area) enables to obtain precise information on lateral diffusion coefficients as well as on the surface concentration of fluorophores. Furthermore, the method allows for proper positioning of the plane of interest in the focus and additionally enables to calculate the width of the focused beam without calibration measurements.
Measurements done by Benda et al were performed on model systems of supported phospholipids bilayers (SPBs). We show that the method can be successfully applied in the cellular experiments carried out in the plasma membrane of OLN-93 oligodendroglial cells and moreover, that it can be even further utilized especially for purposes of the *in vivo* applications.
Eventually, the method helps better characterize the cellular membrane dynamics[2], such as partitioning and confinement of the fluorophor in microdomains, formed due to differences in lipid miscibility, or interactions of the fluorophor with intercellular meshwork.

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P24: **Chromatin organization and dynamics studied by tracing single genomic loci and nuclear bodies *in vivo***

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Clones of the human osteosarcoma cell line U2OS were generated that have *lac*O operator repeats stably integrated at different genomic loci. The sites with *lac*O arrays were visualized by fluorescence microscopy with Lac repressor fused to the auto fluorescent EGFP or mRFP1 domains. This system originally established by Belmont and co-workers allows the study of the conformation and dynamics of a single chromatin locus *in vivo*. We selected a single clone with three distinct inserts that can easily be differentiated by their apparent size in the interphase nucleus. The precise chromosomal integration locus was determined by FISH and mFISH to be on chromosomes 6, 11 and 12. The mobility and relative nuclear location of these three different loci is studied to addresses the following points:
The type of movements of chromatin is characterized on the time scale of seconds, minutes and hours. The mobility of different loci is compared within the same cell.
The chromatin mobility is correlated with the mobility of nuclear Cajal and PML bodies labelled with Coilin-EGFP or PML3-EGFP. It is tested if the accessibility and dynamics of chromatin determine the location and movement of these bodies as proposed previously according to the "moving coral" model (Görisch et al., Proc. Nat. Acad. Sci. USA 36, 13221-13226).
The dynamic positioning of the Lac repressor labelled chromosomes 6, 11 and 12 with respect to each other and to their location in the nucleus (center, periphery) is studied over time. The cell cycle state is determined by co-transfection with PCNA-EGFP.

P25: **Visualisation of interactions in living cells using FLIM and FRET**

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In order to understand functional changes within living neurons (e.g. during synaptogenesis), a precise investigation of the molecular interactions between macromolecules within their natural environment is a prerequisite. Previous investigations for studying the dynamics within living cells were based on the use of video or confocal laser scanning microscopy in combination with vital fluorescent markers. Unfortunately, most of these visualisation techniques (fluorescence/CLSM/2-Photon, etc) use high illumination intensities that cause considerable photo-dynamic reactions within living cells and, therefore, do not allow a non-distorted continuous observation of dynamic processes for a longer period. We were successful in creating a microscopic set up with ultra sensitive detectors like DL (Delay Line) and QA (Quadrant Anode) for studying interactions in living neurons by a combination of FLIM and FRET.
A ratiometric chloride indicator, Clomeleon was used initially to study FRET in living neurons. In Clomeleon, a chloride sensitive variant of YFP called Topaz was linked with a relatively chloride insensitive CFP by using a 24 amino acid linker. We studied the fluorescence dynamics of Clomeleon in hippocampal cell cultures at three different stages of maturation (DIV 7, 10 and 15). In young hippocampal cells (DIV 7), we found a quenching of the YFP moiety of Clomeleon in the majority of neurons, indicating a reduction or absence of energy transfer which might be due to the high intracellular Cl^- concentration present. In the majority of mature cells, quenching of YFP seemed highly reduced, due to better energy transfer from CFP in presence of lower Cl^- concentration. Analysis of the Decay Associated Spectra (DAS) corroborated the presence of an energy transfer with a negative pre-exponential factor corresponding to the lifetimes participating in the energy transfer.
Bassoon is among the earliest proteins to be incorporated into nascent synapses, consistent with its role in active zone assembly. Despite their exquisite localization in the CAZ (cytomatix at the active zone) and putative role in active zone assembly, little is known about the mechanisms of CAZ assembly and of targeting of these cytomatrix proteins to active zones. Previous studies have shown that these proteins play a pivotal role as scaffolding constituents within the presynaptic boutons of developing synaptic contacts. In order to address this question, we investigated xFP-fusion constructs of the large neuron-specific protein (Bassoon: 420 kDa) and its putative binding partners identified so far including the transcriptional corepressor CtBP1 and Cast1, a member of the ERC family of proteins. We were able to study the interactions of these proteins in heterologous cell lines as well as in dissociated hippocampal cell cultures. A multi-exponential decay analysis was done and a reduction in the average lifetime of the donor was observed in presence of energy transfer. With the current study, we hope to get a better understanding of the different mechanisms underlying targeting of these proteins and synapse assembly.

P26: **Computer modeling of renal glomeruli by means of spatial visualization technique of microvascular networks**

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Glomerular capillary loops are complex vascular filters composed of interdigitating podocytes and fenestrated endothelial cells with an intervening matrix. This arrangement renders the glomerulus difficult to analyze by conventional techniques, especially, when pathologic lesions distort glomerular architecture. Recent advances in three-dimensional multicolor imaging and segmentation of single glomerular components enable to dissect this complex structure with finer detail.
The aim of our study is to present new methodologies for computer modeling of renal glomeruli in light, fluorescent and confocal microcopy.
Material for confocal microscopy studies (Zeiss upright CLSM with 488nm argon laser) of normal rat renal glomeruli was perfusion fixed, then washed in PBS, sectioned onto 200 μm sections and stained with either eosin or acriflavine. Material with pathologic lesions consisted of renal tissue with nephrotic syndrome glomerulopaties in children. Paraffin sections of 6 μm were processed according to ABC technique using monoclonal mouse anti-human antibodies against CD31 and CD34 (Dako). Double immunofluorescence, paraffin sections were incubated with anti-podocalyxin and either CD31 or CD34, and observed in Olympus BX60 microscope.
Modeling of $CD31^+$, $CD34^+$ and podocalyxin positive reaction by using a spatial visualization technique was performed in multicolor images extended to three-dimensional space by introducing the marker intensity as the third dimension. Thus, the markers were represented in form of connected prisms and pyramids, showing their spatial distribution and intensity variance. The amount of positive reaction was quantitatively assessed from the orthogonal projection of markers' solid bodies onto the plane and used for cluster analysis of segmented glomerular components. Spatial image analysis was performed by a computer program designed and programmed in C++ by Strzelczyk. By using the same technique, cross-sections of renal capillaries were segmented from series of epifluorescent confocal images and used for three-dimensional reconstructions of capillary networks.

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The Authors thank Prof. M. Witt (Dresden, Germany) for his help and comments in microscopy studies of vascular markers.

P27: **Lifetime Imaging and FCS Upgrade Package for Commercial Laser Scanning Confocal Microscopes**

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Confocal Laser Scanning Microscopes (LSMs) are widely used tools in biochemistry, cell biology and other related sciences today. However, the capabilities of these microscopes can be further enhanced by adding another dimension: time. The upgrade towards temporal resolution is easily done using picosecond diode lasers along with time-correlated single photon counting (TCSPC) for data acquisition. The pulsed diode lasers are integrated in a special laser coupling unit that allows the combination with the continuous-wave (cw) lasers of the LSM. In this way it is possible to easily switch from cw lasers for typical LSM experiments to pulsed lasers used for FLIM measurements independent of the chosen wavelength. The upgrade allows additional measurement modes, like Fluorescence Correlation Spectroscopy (FCS) or Fluorescence Lifetime Imaging (FLIM).
Our upgrade is based upon a special designed variant of TCSPC, which is the so-called Time-Tagged Time-Resolved (TTTR) mode, that is implemented in the TimeHarp 200 PC-board. Essentially, it is an elegant extension of the classical TCSPC preserving the full photon information content. There is no data reduction done by the electronics, nor limitation by on-board memory, because the records are continuously stored on the computer hard disk. The result is an information-rich TTTR datafile, where virtually all algorithms and methods for the analysis of photon dynamics or lifetime imaging can be applied[1].
In this presentation we demonstrate FLIM as technique for measuring dimer formation of transcription factor proteins in the nucleus of living cells using a FRET approach. Due to the dimer formation the fluorescence of the donor FRET molecule CFP is quenched leading to a decrease in fluorescence lifetime. In another example it is shown that the determination of the relative hydrophobicity inside of hepatocytes (HepG2) is possible using the dye NBD (nitrobenzoxadiazol) as a marker, because the lifetime of NBD acts as an indicator for the hydrophobicity of the surrounding medium. All measurements were performed on an upgraded Olympus FluoView 1000.

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P28: **Dynamic simulation of active and inactive chromatin domains**

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Presently, a rosette like structure of chromatin loops is assumed to exist for the experimentally observed chromosomal foci. On the basis of the 30nm fiber a polymer model was implemented which allows the dynamic folding in rosette like structures when attractive sites were distributed along the fiber.
These attractive sites, representing condensing agents (HMG/SAR, HP1, cohesin, condensin, DNA-DNA interaction etc.), tend to aggregate and form rosettes. The diameter of these rosettes depends on the number of attractive sites and therefore also on the loop size. A crucial question now is the accessibility of these rosettes for transcription factor complexes. Presently there still two opinions about the aggregation process of transcription and splicing factors. They might form functional complexes directly at the sites of genes. Alternatively, (sub-) complexes are built up at distant sites, in the so called inter-chromatin regions, and subsequently reach the genes by passive diffusion. In the later case, such aggregates are in the order between 55 and 70nm in diameter and have to access the active genes to allow transcription. Brownian Dynamics simulations of rosettes/TF complexes were performed to investigate the dynamics of the interaction between the 30nm fibre and the proteine complexes.

P29: **Molecular Interactions of a Receptor/TransducerComplex from *N. Pharaonis* Studied with Fluorescence Spectroscopy**

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Archaebacterial photoreceptors are a family of light sensitive proteins, which mediate phototaxis by regulating cell motility through a two-step signaling cascade. The sensory rhodopsin II (SR II) and the associated signal transducing protein Htr II build a receptor/transducer complex in the archaeal species *Natronomonas pharaonis*. In our experiments we labeled different cystein mutants of SR II and Htr II with maleimid-functionalized fluorophores in order to obtain site-specifically labeled probes that are also suitable for single molecule detection. The labeled proteins were reconstituted into large unilamellar vesicles (LUV, diameter ~ 100-200 nm) with protein-lipid ratios between 1/10 to 1/100 (w/w). Tethered LUVs as well as proteins reconstituted in giant unilamellar vesicles (GUV, diameter 10 – 20 mm) serve as samples for fluorescence spectroscopic studies where we address major questions, concerning molecular interactions of the receptor/transducer complex and light-induced conformational changes upon photoactivtion.

P30: **Cryo-electron tomography of Plasmodium sporozoites**

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Plasmodium sporozoites are the forms of malaria parasites injected by a blood feeding mosquito into the skin of the host. Sporozoites in the skin are highly motile and use a unique motility system that allows them to migrate in order to reach the blood circulation and to invade liver cells[1,2]. The sporozoite constitutes a highly polarized eukaryotic cell with a slightly curved regular cell shape of 10 micrometer in length and 1 micrometer in diameter. We believe that these features make it an ideal model object for cryo-electron tomography (Cryo-ET) and correlated light-electron microscopy (CLEM) approaches. We aim at generating tomograms from all parts of the entire sporozoite and have completed analysis of the most diverse apical (front) end, which contains a large number of different vesicles as well as structures that are thought to serve as microtubule organizing centers. We analyzed in detail the microtubules from sporozoites and found a smaller than usual electron lucent lumen indicating a different topology of the microtubule wall when compared to microtubules from other eukaryotic cells. Currently, we expand these observations to the related parasite Toxoplasma gondii.

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P31: **Coumarinylmethyl esters for ultrafast release of high concentrations of cyclic nucleotides upon one- and two-photon photolysis**

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Caged compounds (biologically inert photoactivatable precursors) of cyclic nucleoside monophosphates (cNMPs) are powerful tools for studying spatiotemporal dynamics of cyclic nucleotide-dependent processes. Among these compounds, (coumarin-4-yl)methyl esters of cNMPs are most useful. They show no background bioactivity, are stable to solvolysis, and photolyze efficiently and extremely quickly.[1,2]

The combination of high solubility in aqueous buffer and high photosensitivity allows large and instantaneous concentration jumps of cNMPs upon flash photolysis both in solutions and inside living cells. Furthermore, we show that the new caged compounds photorelease the cNMPs upon one- and two-photon excitation. An additional advantage of these compounds is the possibility to determine quantitatively the amount of the photoreleased cyclic nucleotide inside cells using fluorescence measurements.

At least, the novel photoreleasable BCMACM protecting groups should be useful in caging and uncaging of other biomolecules with phosphate, carboxylate, carbonate or carbamate functionalities..

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P32: **Estimation of minimal distances between colocalized genes in *Drosophila melanogaster***

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Our lab is interested by the nuclear organization of specific target genes regulated by the chromatin factors of the Polycomb group in *Drosophila melanogaster*. Two of these genes are *Antp* and *Abd-B*, two Hox genes that are separated by 10Mb on chromosome 3.
Using a two color Fluorescence *in situ* Hybridization (FISH) protocol and widefield fluorescence microscopy, we observed a significant number of colocalization events between *Antp* and *Abd-B* in cell nuclei of approximately 5 micrometers diameter. Unfortunately, optical resolution limits and chromatic aberrations induce weak precision of distance measurements between our two 30kb FISH probes.
In order to improve the precision of colocalized *Antp-Abd-B* distance measurements, we use a combination of fluorescence microscopy and image analysis techniques. For analysis, signal centroid calculation at 200nm Z axis sampling rate allows sub-resolution localization of segmented objects. To estimate the quality of acquisitions, each one is repeated two times and the corresponding centroid positions are compared. Chromatic aberration calibration of our optical setup is done using multi-fluorescent 200nm beads. Fitting linear functions with these calibration data leads to chromatic correction parameters. Remaining *in situ* chromatic deviation is estimated by distance measurements on corrected signals of a single *Antp* probe revealed with two colors. Finally, the closest distances (≤ 300nm) between *Antp* and *Abd-B* are measured and analyzed.
Preliminary results show that the 10 Mb separated *Antp* and *Abd-B* Hox genes occasionally colocalize at distances that are compatible with chromatin fiber contacts.

P33: **Dynamics of DNA methyltransferases in living cells**

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DNA methylation plays a central role in the regulation of gene expression, chromatin condensation and genome stability. We found that Dnmt1 shows a complex and cell cycle dependent distribution. It associates with the replication machinery during S phase and with constitutive heterochromatin from late S till M phase [1]. In contrast, Dnmt1 is retained in the cytoplasm during early development which may contribute to passive demethylation and epigenetic reprogramming [2]. Recently, we have developed an assay to measure and study DNA methyltransferase activity in living cells using live cell microscopy and photodynamic techniques including fluorescence bleaching and photoactivation assays [3].

In mammalian cells the replication of genetic and epigenetic information is directly coupled. However little is known about the maintenance of epigenetic information in DNA repair. Using a laser microirradiation system to introduce DNA lesions at defined subnuclear sites we tested whether DNA methyltransferases are recruited to sites of DNA repair in vivo. Time-lapse microscopy of microirradiated mammalian cells revealed that Dnmt1 and PCNA accumulate at DNA damage sites. Deletion analysis showed that Dnmt1 recruitment was mediated by the PCNA-binding domain (PBD). These data point to a direct role of Dnmt1 in the restoration of epigenetic information during DNA repair [4].

Currently, we are studying inhibitors, interactions and mutants of Dnmt1 in living cells.

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P34: **Cryo-Electron Tomography of Neuronal Processes and Synapses**

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Cryo-electron tomography (cryo-ET) combines electron-tomography with the cryo-preparation that allows the investigation of frozen-hydrated samples free of artefacts caused by chemical fixation, dehydration, and uneven stain accumulation. It thus allows us to investigate three-dimensional structural features of biological material preserved in a "near-to-physiological" state.

We obtained tomograms of isolated glutamatergic synapses (from the synaptosomal fraction) under different experimental conditions. We have also optimized the cell culture conditions needed to obtain mature neuronal cultures from hippocampus suitable for cryo-ET, and obtained tomograms that show many known structural features of axonal and dendritic processes. These include cytoskeleton, postsynaptic density, and internal membranous compartments such as synaptic vesicles, transport vesicles, mitochondria, and smooth endoplasmic reticulum. This is the first time that these structures are observed in frozen-hydrated state within their native, cellular environment, in three dimensions.

We also improved our image processing methods in order to increase the usable resolution and analyze the structures present in our tomograms. To this end we optimized parameters in denoising algorithms developed for cryo-tomograms, and improved our parameter-free automated procedure for segmentation and morphological analysis of molecular complexes.

P35: **Protein dynamics: novel fluorescence probes for single molecular spectroscopy**

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Single Molecule Spectroscopy (SMS) represents a major technique in understanding biological processes on a molecular level and is used for the characterization of biomolecules and their specific interactions in biological samples.

To observe individual molecules, it is essential to use sufficiently dilute sample solutions and to reduce the detection volume to within subfemto liter range. Because of its outstanding selectivity and sensitivity, fluorescence is the method of choice for the detection in SMS. For the investigation of intra-molecular and diffusion-controlled processes on a μs to ms time scale fluorescence correlation spectroscopy (FCS) has proven successful.

Therefore, fluorescence probes with specific characteristics, such as high stability and sensitivity for environmental parameters, and high fluorescence quantum yield are highly needed. By coupling onto biomolecules like biotin, fluorescence probes can deliver information about the localization and mobility of specific molecules within living cells as well as conformational changes of macromolecules.
New fluorescence probes of the cyanine type, their biotin derivates and the corresponding biotin streptavidin complexes were characterized. Time-resolved anisotropy was used to monitor the binding between biotin and streptavidin.

Because of their red to near-infrared excitation, the investigated probes are especially suited for the characterization of biological samples. Diffusion coefficients of these fluorescence probes and their biotin derivates were determined by means of FCS.

P36: **HIV-1 RNA Dynamics in the Infected Cell's Nucleus**

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Genome function is regulated also by the spatial arrangement of the genes with respect to gene expression processes in the nuclear space. Retroviruses such as the human immunodeficiency virus (HIV-1) integrate their genome in the host cell's chromatin where they can remain transcriptionally inactive. Transcriptional activation of the viral promoter LTR depends both from host cell's factors, the viral Tat trans-activator and the chromatin environment at the site of integration[1].
In order to study the activation of HIV-1 gene expression from the integrated viral LTR we exploited a method that allows the visualization of newly transcribed RNA in living cells through the specific recognition of an array of RNA consensus sequences for the bacteriophage MS2 coat protein tagged with YFP[2]. We observed that transcription of HIV-1 occurred in discrete foci within the cell's nucleus. These foci, representing newly transcribed RNA, co-localized with the viral Tat transactivator. Localization of HIV-1 RNA depended from the site of RNA tagging: intronic RNA remained nuclear whether exonic RNA was exported to the cytoplasm. However, in the presence of the Rev protein, also the HIV RNA tagged within the intron was exported from the cell's nucleus mimicking the Rev-mediated export of genomic viral RNA.
This experimental setting was used to measure the dynamic of HIV-1 RNA transcription in living cells. By fluorescence recovery after photobleaching (FRAP) we were able to demonstrate that the process reaches a steady state within seconds and with a negligible immobile fraction. Inhibition of transcription by actinomycin D dramatically increased the immobile fraction of the FRAP and reduces the number of foci visible in the cells' nuclei.
In summary, we have developed a method to visualize HIV-1 RNA that will enable us to study the dynamics of HIV-1 transcriptional activation in living cells.

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P37: **Labelling of nuclear structures in living cells**

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Live cell fluorescence microscopy experiments often require the visualization of the nucleus to determine the nuclear morphology or the localization of nuclear compartments like chromatin and nucleoli. We compared five different DNA dyes, TOPRO-3, TOTO-3, propidium iodide, Hoechst 33258, and DRAQ5, for their usefulness in live cell experiments using widefield epifluorescence and confocal laser scanning microscopy. Furthermore, we developed and tested a transducible fluorescent marker for the nucleolus in living cells.
The DNA labeling ability of the different dyes was compared to fluorescent histones as an independent fluorescent chromatin label. From the dyes tested, only Hoechst and DRAQ5 could be used to stain DNA in living cells. However, DRAQ5 had several advantages, namely very low photobleaching, labelling of the chromatin comparable to H2B-GFP fusion proteins, and fluorescence properties compatible with genetically encoded fluorescent proteins such as C/G/YFP or mRFP [1].
The nucleolar labeling consists of a short basic peptide fluorescently labeled, which combines the ability of membrane penetration in living cells and high affinity to the nucleolar compartment [2]. The fluorescent peptide marker can be used in combination with different fluorophores like fluorescent proteins or DNA dyes and nucleolar labeling is preserved during fixation of the cells. Furthermore, we observed a high stability of the label in the nucleolus in long term studies over 24 h as well as no effect on the cellular viability and proliferation.
Both DNA and nucleolar labels could be used in a variety of cells including primary cultures and the labelling efficiency was over 95 % after a few minutes incubation in the culture medium. Therefore, they are valuable molecular tools for cell biology that allow a fast and non invasive labeling as well as microscopic visualization of these structures in living cells.

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P38: **Manipulating chromatin condensation states and its consequences for the dynamic nuclear architecture in living cells**

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The study of structure and function of the cell nucleus revealed a highly organized organelle storing and realizing genetic information. Although there are no separating membranes, the nucleus is highly compartmentalized for different functions in nucleic acid metabolism. The nuclear DNA is organized together with structural proteins into a dynamic chromatin structure reflecting and controlling changes in gene expression during the cell cycle and cellular differentiation. Chromatin subsets are therefore also termed eu- and heterochromatin according to their condensation state, transcriptional activity and modifications of chromatin organizing proteins. Euchromatin is generally assumed to be actively transcribed and less condensed while heterochromatin is considered transcriptional inactive with a condensation state similar to mitotic chromosomes [1, 2, 3].
It is still unclear, how changes in the chromatin condensation state influence nuclear architecture and the mobility of chromatin organizing proteins. To address these questions, we analyzed how various macromolecules access or are excluded from chromatin in different condensation states [1, 2, 3]. To probe the dynamic properties of the nuclear structure we used fast multicolor live cell imaging and fluorescence photobleaching of fluorescently labeled proteins and DNA dyes [4]. In particular, we analyzed the accessibility of different chromatin subsets in combination with treatments, inducing alterations on the condensation level of chromatin. Simultaneously, we tested the influence of manipulating chromatin condensation on the binding of DNA metabolic enzymes and various chromatin factors.
With these studies, we gained insight into principles of the functional nuclear organization by directly evaluating the influence of different functional chromatin compaction states on the protein accessibility in one and the same living cell.

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P39: **SMI Microscopy using one objective lens**

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The SMI (Spatially Modulated Illumination) Microscope is a widefield fluorescence microscope featuring axially structured illumination, which gives access to information on fluorescent objects with sizes below the classical resolution limit (about 200nm laterally and 450nm axially). Presently, this method is applied to the quantitative analysis of fluorescently labeled biological nanostructures inside three dimensionally intact cell nuclei.
To date, the structured illumination was achieved by focusing two coherent laser beams into the back focal planes of two opposing objective lenses. In a new, more compact setup this is simplified by replacing one of the objective lenses with a mirror which reflects the light and creates by interference the illuminating standing wavefield. We demonstrate the potential of this method for the study of smallest biological structures by a comparison with the results obtained with the conventional SMI Microscope as well as a nanostructure analysis of the transcriptional activity of single, fluorescently labeled gene regions in human cell nuclei.

P40: **3D Imaging of fluorescence patterns in Drosophila melanogaster with OPT**

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3D imaging of fluorescent markers for gene expression in-vivo in *D. melanogaster* as a model organism for understanding many molecular and cellular mechanisms[1,2] in all stadiums of its life cycle would represent a powerful tool in modern genetics.
Our aim is to use optical projection tomography *(OPT)* [3-6] in fluorescence mode to quantitatively reconstruct fluorophore concentration in opaque organs such as the adult head of *D. mel*. To that end, a modified radon transform that takes into account the isotropic emission of the fluorophores has been developed [7].
GFP expression patterns of transgenic *D. mel.* in various life cycle stadiums were imaged and reconstructed using a custom made CCD based microscope setup. Excitation was generated with a halogen lamp using specific excitation filters, and the specimen rotated using a capillary tube and index-matching liquids as in [4]. In order to account for the point source propagation of the fluorescence intensity, a novel modification of the radon transform was employed[7]. The accuracy of the approach was verified with phantom measurements, which yields good comparison with the novel theoretical approach[7]. This approach is also used to image several organs in Drosophila at several stages, reaching good results, which we compare with those obtained using the standard radon transform [3-6].
To obtain quantitative fluorescence images, a modification to the standard radon transform which takes into account the isotropic emission of the fluorophores is essential. The accuracy of this approach has been shown both with phantom and measurements in drosophila melanogaster.

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P41: **Analysis of the recruitment of genetic and epigenetic repair factors in living cells**

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In mammalian cells the replication of genetic and epigenetic information is directly coupled, however little is known about the maintenance of epigenetic information in DNA repair. We employed a confocal laser scanning microscope to locally generate DNA damage at preselected subnuclear sites in BrdU-sensitized cells. Usage of GFP- and RFP-tagged proteins allowed us to directly compare the recruitment of different repair factors to laser-induced DNA damage sites within a single living cell. Time lapse microscopy of microirradiated mammalian cells expressing GFP-tagged Dnmt1, Dnmt3a or Dnmt3b1 together with RFP-tagged-PCNA revealed that Dnmt1 and PCNA accumulate at DNA damage sites as early as 1 min after irradiation in S and non S phase cells, while recruitment of Dnmt3a and Dnmt3b was not observed. Deletion analysis showed that Dnmt1 recruitment was mediated by the PCNA-binding domain (PBD). These data point to a direct role of Dnmt1 in the restoration of epigenetic information during DNA repair[1]. We are currently investigating the kinetics and recruitment mechanism of other factors involved in the recognition and repair of genetic and epigenetic damages in living cells.

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P42: **Subcellular compartmentalization contributes to the microtubule regulating function of spastin**

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Hereditary spastic paraplegia (HSP) is a group of clinically and genetically heterogeneous diseases characterized by neuronal degeneration. The most common cause of autosomal dominant HSP is mutation of the gene encoding spastin. Spastin is an AAA-type family member of ATPases that regulates microtubule dynamics in the cytoplasm. Several spastin isoforms have been described, but their contribution to specific spastin functions is not known. We have investigated the localization, in vivo dynamics, and microtubule-severing activity of two ubiquitously expressed spastin isoforms using confocal microscopy, fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS). These analyses reveal that the longest spastin isoform is an exclusive cytoplasmic protein while a shorter isoform lacking the first 86 aa resides in the nucleus as well as in the cytoplasm. Nucleocytoplasmic traffic of this shorter isoform is slow ($t_{1/2}$ >10 min) indicating an active nuclear retention mechanism. Interestingly the short isoform exhibits a significantly higher microtubule-severing activity than the full-length protein. The regulation of spastin by isoform-specific expression and localization may help in understanding additional aspects of this proteins' function and in fully linking mutations in spastin to HSP pathology.

P43: **Quantitative analysis of Plasmodium sporozoites motility**

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Plasmodium sporozoites form in parasitic cysts at the gut wall of an infested Anopheles mosquito. After release from these cysts, they invade the salivary glands from where they are transmitted into the skin of the host during the bite of a mosquito. Sporozoites move slowly within salivary glands but speed up considerably once injected into the skin, where they move to invade blood and lymph vessels [1-3]. We aim to understand the role of motility during transmission and the biophyiscal basis of sporozoite movement. To gain insights into transmission we image Plasmodium sporozoites that express the green fluorescent protein in their cytoplasm within living mice after natural transmission. Quantitative analysis shows how sporozoites modulate their speed and move with distinct patterns in different environments. For a better understanding of the mechanical forces applied by the parasite during motility to its substrate we use traction force microscopy. For this, sporozoites are isolated from the salivary glands of mosquitoes and imaged on flexible sheets of polyacrylamide gels imbedded with fluorescent beads [4].

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P44: **Imaging spatial and temporal interaction profiles of proteins in living cells using FRET-FLIM**

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Resonance Energy transfer (RET) using the Fluorescence Life Time Imaging (FLIM) is an elegant tool to understand the interaction of proteins in the subcellular compartments below the limit of optical resolution. It has been also used successfully to understand the conformational changes of macromolecules and alterations in the intracellular micro environments. Here we have used multi exponential model to study FRET where we study the contribution of independent decay components to understand the contribution of the lifetimes along the wavelength. Simulteneous detection of donor and acceptor was possible with the help of a novel method called Time and Space Correlated Single Photon Counting (TSCSPC), we are able to record the spectrum and lifetime (using DL Detector) and image and lifetime (using Quadrant Anode Detector).

With the present system, energy transfer dynamics in modified constructs of the ratiometric chloride sensor Clomeleon (Kuner and Augustine 2001) were studied. The fluorescent proteins CFP and Topaz (a chloride sensitive variant of YFP) are separated by different spacers (8aa, 16aa, 24aa respectively). The changes in transfer times were observed by measuring different lifetime components involved and plotting the Decay Associated Spectrum (DAS). The Decay Associated Spectra were plotted for all the constructs by analysing the respective intensity decays with Global analysis software. We were able to experimentally support the structure of the different constructs as predicted by molecular modelling.

As biologically relevant issues we have addressed the folding of the protein non receptor tyrosine kinase LCK in T Lymphocytes by tagging the CFP and YFP at different regions of the protein. LCK is among the first proteins to be recruited to an immunological synapse. From our results it is believed that in the resting T-Lymphocytes the LCK exists in a closed passive conformation because of the intramolecular interactions with SH2 domain and C-terminal tyrosine residue. The structural prediction from the FRET-FLIM studies is compatible with the published and predicted structures of other proteins in the same family of Src Kinases. We have studied the dynamics of the LCK conformational change over time with the stimulation of T lymphocytes with soluble antibodies. Initial results indicate an opening of the conformation to an active state indicated by an increase in the average lifetime in the initial 1-5 minutes from the stimulation and in later stages going back to the closed passive conformation in about 15-20 minutes indicated by the reduction of average lifetime back to the FRET level which was similar before the stimulation of resting cells. This method of FRET-FLIM studies will enable us to understand the spatial and temporal interaction profiles of interaction patterns of macromolecules maintaining the living state of the system

P45: **Probing lipid raft and membrane protein orientation directly by polarized total internal reflection fluorescence (TIRF) microscopy in model membranes**

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Biological membranes are thought to contain specialized domains, variable in size, composition and spatial arrangements of the constitutive lipids and proteins. Data suggests these domains, or "lipid rafts", are critical to several cellular processes including signal transduction, transport, apoptosis, and protein sorting[1]. Dynamic transformations in lipid and protein orientation can occur within a lipid raft or at the liquid-ordered liquid-disordered domain boundary as a result of alterations in membrane composition, temperature, and pH. The role these changes in molecular orientation might play in the function of lipid rafts remains unclear, however. We are addressing this question by exploiting the unique light polarization properties present in TIRF microscopy[2]. A custom built polarized TIRF microscope was used to photo-selectively excite and spatially localize embedded membrane dye molecules with a definite dipole moment relative to the membrane normal in planar substrate-supported model lipid bilayers containing varying amounts of 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC), 1,2-Distearoyl-sn-Glycero-3-Phosphocholine (DSPC), and cholesterol under native-like conditions. Our results demonstrate the existence of sub-domains of higher lipid packing order within rafts doped with the fluorescent indocarbocyanine dye molecule $DiIC_{16}$ (Figure 1).

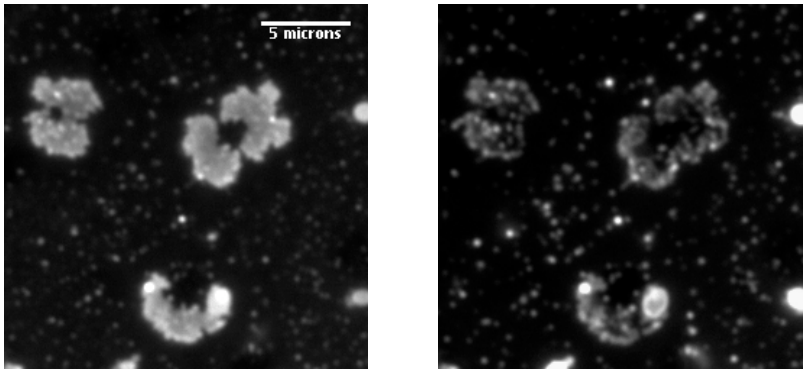

Figure 1: A mixed bilayer containing 50/50 mol% DOPC/DSPC doped with a small amount of $DiIC_{16}$ on a mica substrate imaged by substrate parallel polarized (*s*-pol) light (left) and substrate perpendicular polarized (*p*-pol) light (right). Exposure time = 500 ms. Time in between exposures = 12s.

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P46: **Associations of inner kinetochore proteins**

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DNA segregation of mammalian cells is an essential cellular process mediated by the centromere/kinetochore complex. This complex is located at the primary constriction of the chromosome and consists of centromeric DNA and the overlaying protein structure, the kinetochore. Malfunction of this complex results in aneuploidy and can cause cancer.
Previous studies suggest that a central protein core consisting of the inner kinetochore proteins CENP-A, -B, -C, -H, -I, Hec1 and hNuf2 is present at the centromere during the whole cell cycle and mediates the connectivity between the centromeric DNA and proteins from the central and outer kinetochore. This central core seems to be necessary for the recruitment of the further kinetochore proteins and is therefore essential for a functional kinetochore structure.
We determined the detailed protein assembly and the protein neighborhood relations of the kinetochore complex. We measured the associations within the inner kinetochore by applying acceptor bleaching FRET (Fluorescence Resonance Energy Transfer) and FLIM (Fluorescence Lifetime Imaging) *in vivo* to define in detail the associations between these inner kinetochore proteins in their natural setting, the living cell. Until now, *in vitro* efforts could not elucidate the correct molecular composition of this complex. Many of the proteins involved in kinetochore formation are known, however, little information is available on their molecular structures and the complex architecture although structural phenomena seem to play an important role for kinetochore complex formation and function.
Our analyses elucidate the kinetochore complex architecture and the protein interdependencies of its assembly. These results will contribute to our molecular understanding of mitosis and how particular kinetochore protein interactions might be associated with aneuploidy in mammals.

P47: **Fully integrated light and atomic force microscopy to study cellular structure and adhesion**

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The atomic force microscope (AFM) is a mechanical microscope, where contrast during imaging is generated depending on the structural properties of a sample. When imaging cells, the heterogeneous nature of the cell surface means that it can be difficult to interpret the significance of structural data from the atomic force images alone. The combination of AFM with light microscopy extends the potential for this imaging technique. To be truly effective however, not only must the AFM be integrated into an inverted light microscope, but the imaging software must be able to accurately correlate a field imaged using the AFM with an optical image acquired from the light microscope. New software for the JPK Nanowizard™ has been developed that can use tip location to map an optical image into the AFM image space, made possible by the fact that the instrument uses closed loop positioning of the AFM tip. This means that, tip position for imaging, force-spectroscopy measurements and manipulation, can now be chosen with respect to the integrated optical image.

P48: **Time-correlated single photon counting by improved quadrant-anode position sensitive photo-multipliers**

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Studying protein-protein interactions in living cells requires new approaches and instruments in time-resolved spectroscopy. Time correlated single photon counting (TCSPC) is based on principle of detecting the individual photons emitted by the pulse source. The greatest advantage of this techniques is the possibility of measuring the time distances between the laser pulse and the reemitted photon as precise as 100 ps (100 x 10^{-12} seconds) which allows one to measure dynamic processes in a living cells. The time- and space- correlated single photon counting (TSCSPC) instrument opens a new field of the biological research providing spatial information for each individual photon along with timing. The basic operational principle of the quadrant-anode micro-channel plates based photomultiplier (QA) is the following: (i) an initial photon being emitted by the sample hits out the electron from the photocathode (ii) the electron carried by the electric field comes to the input surface of the MCP and results an electron avalanche (iii) that falls to the space sensitive anode system; (iv) the amounts of charge from each quadrant are strongly depend on the coordinate of the initial photon. Using mathematical algorithms it is possible to calculate the position of the initial event by measured charges. The advantage of the simultaneous measurement of the coordinate and time is the ability to select the region of the interest and to acquire single molecule tracking information. The major limitation of present TSCSPC system was an insufficient throughput that only allowed to study slow dynamic changes. Therefore, recently the computer interfacing part was improved to reach 100 KHz count rate and existing algorithms of the real-time processing of the QA were updated. Additionally to the visualisation at extremely low excitation levels the new configuration now allows to study faster dynamic processes in living cells.

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P49: **Single molecule microscopy using focal plane illumination**

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Single molecule fluorescence microscopy performed in spatially extended samples suffers from the drawbacks of a low axial resolution, high background light and rapid photo-bleaching[1]. To overcome these disadvantages we use a focal plane illumination instead of the standard epi-illumination. We create a 20x20µm wide and 2µm thick light sheet within the focal plane by a custom made cylindrical lens system (NA = 0.33). In this manner an optical sectioning microscope is created [2,3]. The light sheet is produced inside a water chamber, where the sample is fixed within an agarose gel cylinder [2]. Fluorescence light is detected perpendicular to the illumination plane by a water-dipping microscope objective lens (60X, NA = 1.1) and imaged onto a CCD camera. With this setup, only the plane of interest is illuminated and therefore affected by photo-bleaching. Excitation of the fluorescence light in the focal plane only leads to a background reduction. The axial resolution is determined by the light sheet thickness and the resolving power of the detection objective lens. The penetration depth of the optical sectioning is limited by the working distance of the water-dipping microscope objective (w.d. = 2.5mm). With this setup we achieve an axial resolution of approximately 830nm and a significant reduction of fluorescence background in single molecule imaging in extended 3-dimensional systems such as cell nuclei.

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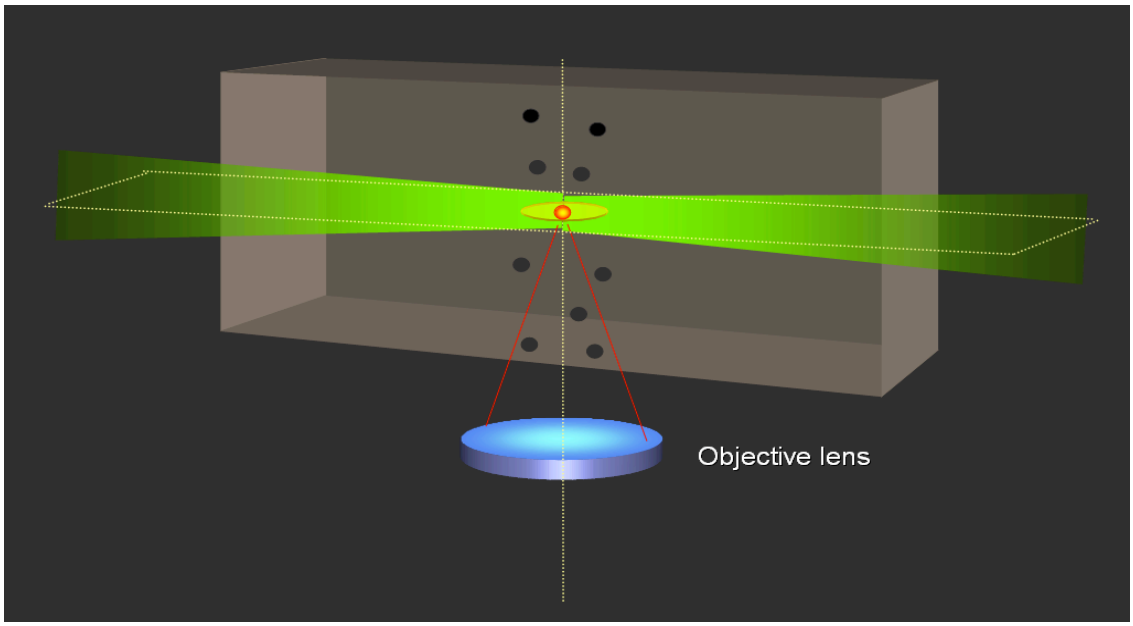

Schematic view of the focal plane and perpendicular detection of the fluorescence light

P50: **Quantitative single pair FRET by pulsed interleaved excitation and fluorescence correlation spectroscopy**

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With recent advances in sensitive fluorescence detection techniques single pair Förster resonant energy transfer (spFRET) is of increasing interest to detect e.g. co-localization of molecules or to measure conformational changes on the level of single molecules. However, in spite of the strong dependency of the energy transfer efficiency upon donor-acceptor separation quantitative results are barely to find, i.e. FRET experiments are interpreted qualitatively mainly.Quantitative analysis of spFRET is often hampered by a zero efficiency peak occurring in the FRET efficiency histogram, caused by molecules with missing or non fluorescent acceptor. Further problems arise from the presence of crosstalk due to imperfect spectral filtering, direct excitation of the acceptor as well as not directly measurable excitation- and quantum-efficiencies of the fluorophores and sensitivities of both detection channels.
To overcome the above-mentioned difficulties we applied[1] (dual color) pulsed interleaved excitation in FRET measurements (PIE-FRET) combined with time correlated single photon counting. Events contributing to the zero efficiency peak have been identified and eliminated to obtain clean FRET-histograms. Since direct acceptor excitation, molecular brightness of donor and acceptor fluorophores as well as crosstalk or leakage was determined by analyzing the same data-set with fluorescence correlation spectroscopy (FCS), all quantities required to analyze FRET-measurements properly were available. The advantages of the PIE-FRET approach were demonstrated with a poly-proline assay labeled with Alexa 555 as donor and Alexa 647 as acceptor, respectively.

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[1] Rüttinger S., Krämer B., Roos M., Hildt E., Koberling F., and Macdonald R., (2006) J.Biomed.Opt. 11 (2), 024012

P51: **Combining spectroscopic methods for investigation of single autofluorescent proteins on the nanoscale**

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Autofluorescent proteins are a field of great interest in various branches of biology and chemistry. Since they can serve for example as in vivo probes on the single-molecule scale a deeper understanding of the underlying photophysical processes is of great importance.

We present the combination of various optical techniques in the single-molecule regime to characterise spectroscopic properties of individual autofluorescent proteins, such as the bichromophoric DsRed mutant E8.
Using high resolution confocal microscopy investigation of structures on the nanoscale is accessible. In combination with high detection efficiency, ultra sensitive oberservation of individual protein entities is feasible.
Thus single molecule fluorescence studies give insights to the composition of tetrameric subunits[1] whereas surface enhanced Raman spectroscopy (SERS) allows for characterisation of binding properties of the proteins on metallic surfaces.[2]

Embedding the proteins between the mirrors of a microresonator can help to investigate fluorescence resonance energy transfer (FRET) within individual protein entities. Here we present a novel microcavity design based on a Farbry-Perot type interferometer[3] which allows for manipulation of both fluorescence and Raman emission of embedded autofluorescent proteins. Using time correlated single photon counting techniques FRET activity can be investigated due to changes in the radiative emission rate of the proteins.

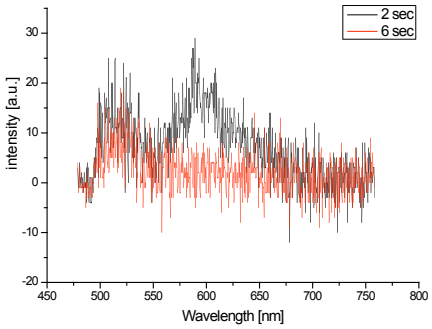

Fig 1: Single protein fluorescence spectrum showing selective photobleaching of one chromophore

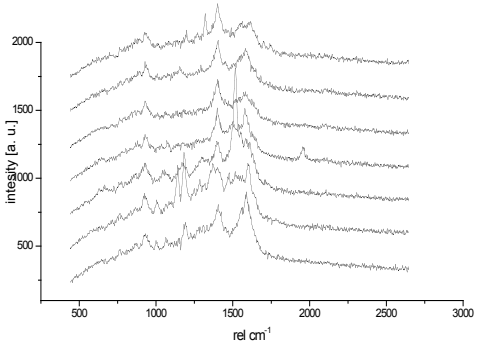

Fig 2: Series of Raman spectra of one single protein. High dynamics of single Raman lines are visible

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P52: **Ultra-high resolution light microscopy using structured illumination**

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Studies of subcellular structures using state-of-the-art light microscopy have been restricted by the diffraction barrier of optical resolution that is 200-300 nm in the xy-plane and 500-800 nm along the z-axis. Recent advances have made possible to surpass the diffraction limit in axial direction using 4Pi microscopy [1] and additionally in lateral direction when combined with stimulated emission depletion (STED) microscopy [2]. An alternative approach developed at the UCSF uses the principle of structured illumination (SI) to improve lateral as well as axial resolution by a factor of two below the diffraction limit [3,4]. This technology has been implemented in a specially designed microscope platform, termed OMX, which provides unprecedented sensitivity and mechanical stability.
To explore the potential of the SI technology we have tested the OMX prototype on a wide variety of biological structures. We show here the conceptual basics of the OMX microscope and present exemplary high-resolution data on various structural features in mammalian cell nuclei. We show for the first time multi-fluorescence 3-dimensional (3D) data on the ultra-structural organization of the nuclear envelope and chromatin. In addition, we provide first light microscopical evidence for the organization of 300-800 nm sized DNA replication foci into smaller subunits of ~120 nm size. These results clearly demonstrate the potential of the OMX microscope for multi-wavelength 3D-imaging of biological samples with subdiffraction resolution that will allow new insights in biological structures and will help to narrow the gap between light and electron microscopy.

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P53: **Tracking of stem cell behavior at the single cell level: new tools for old questions**

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Despite intensive research, many long-standing questions of hematopoietic stem cell biology remain unsolved. One major reason is the fact that hematopoiesis is usually followed by analyzing the fate of populations of cells – not of individual cells - at very few time points of an experiment, and without knowing (or quickly loosing) their individual identities. The static picture yielded by this approach makes it impossible to appreciate the dynamic developmental processes leading to the (re)generation of the blood system from single hematopoietic stem cells (HSCs). Real-time tracking of individual cells in culture, tissues or whole organisms would be an extremely powerful approach to fully understand the developmental complexity of hematopoiesis. However, many of the needed tools are still under development and their application in stem cell research remains difficult. Here, a computer aided incubation and bioimaging system was developed to follow the fate of individual cells over long periods of time (up to several weeks) with highest temporal resolution. New software modules for data acquisition and analysis were written, helping to control microscopes and record, display and statistically analyze the divisional history, position, properties, lineage choice etc. of all individual cells derived from HSCs. In combination with newly generated transgenic mice expressing different sub-cellular forms of fluorescent proteins in specific hematopoietic lineages, this system is used to analyze the embryonal generation of blood cells and the behavior of individual adult HSC and all of their progeny over many generations at the single cell level.

P54: **Inversion techniques in structured illumination fluorescent microscopy**

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Recently, fluorescent microscopy with structured illumination has been proposed as an alternative to classical confocal microscopy [1,2]. In this technique, the sample is illuminated by a light-grid and the fluorescence is detected with a standard objective. The position of the grid is moved several times (in general 5 positions are necessary) in order to illuminate the object in a uniform way. For each position of the grid, an image of the fluorescence is recorded. Then, a numerical treatment, based on Fourier Transform is performed on the data to reconstruct the image of the whole sample [3]. In a classic confocal microscopy, the sample is illuminated by a localised spot and the detection is performed in a localised volume. Moving in parallel both the illumination spot and the collection volume permits the building of a map of the sample. The advantage of structured illumination microscopy compared to classical confocal microscopy is that it requires less mechanical manipulations to reconstruct the whole sample. In this work, we study theoretically the power of resolution of the classical confocal microscope and that of the structured illumination microscopy. We show that, basically, these two systems present the same power of resolution as long as proper deconvolution is performed. We then propose a numerical technique, based on the inversion algorithms that prevail in micro-wave imaging, which permits to reconstruct the image of the sample from the recorded data [4] in structured illumination microscopy. We study the interest of such a method, as compared to the classic Fourier Transform method, in terms of noise robustness. Another advantage of our approach is that it allows one to incorporate easily a priori informations in the inversion procedure that may improve further the resolution of the image [5].

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P55: **The SNARE-motif is essential for the formation of syntaxin clusters in the plasma membrane**

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In the plasma membrane, syntaxin 1 and syntaxin 4 clusters define sites at which secretory granules and caveolae fuse, respectively. It is widely believed that lipid phases are mandatory for cluster formation, as cluster integrity depends on cholesterol. We found that the native lipid environment is not sufficient for correct syntaxin 1 clustering and that additional cytoplasmic protein-protein interactions, primarily involving the SNARE-motif, are required. Apparently no specific co-factors are needed because (i) clusters form equally well in non-neuronal cells and (ii) as revealed by nanoscale subdiffraction resolution provided by STED-microscopy, the number of clusters directly depends on the syntaxin 1 concentration. For syntaxin 4-clustering the N-terminal domain and the linker region are also dispensable. Moreover, clustering is specific because in both cluster-types syntaxins mutually exclude one another at endogenous levels. We suggest that the SNARE-motifs of syntaxin 1 and 4 mediate specific syntaxin-clustering by homo-oligomerization, thereby spatially separating sites for different biological activities. Thus, syntaxin-clustering represents a mechanism of membrane patterning that is based on protein-protein interactions.

P56: **Intranuclear Dynamics and Mobility of Balbiani Ring mRNP Particles in Living *Chironomus tentans* Salivary Glands**

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The salivary glands of the dipteran *Chironomus tentans* provide an elegant model system for the analysis of specific messenger ribonucleoprotein particles, the Balbiani Ring (BR) mRNP. BR mRNP particles contain long RNA transcripts of roughly 35-40 kb in size which possess a highly repetitive sequence. The diameter of the granular BR mRNP particles is around 50 nm and their geometry was meanwhile characterised by Electron Tomography. Transcription and splicing of the BR mRNA and the formation of the BR Particles is genetically and biochemically thoroughly investigated, and in several recent studies their intranuclear localisation in fixed glands was visualized by electron microscopy [1]. However, up to now little is known about the intranuclear dynamics and mobility of the BR mRNP particles *in vivo*. Using a direct kinetic approach we analyse the intranuclear diffusion of BR particles by single particle tracking of fluorescence labelled BR mRNPs in living cells. Labelling is achieved *in situ* by nuclear microinjection of Cy-5-conjugated oligonucleotides which are complementary to the highly repetitive sequence on the BR mRNA. This approach generates fluorescent particles *in vivo*. Injection of control oligos proves that this labelling is specific. Using high-speed laser microscopy we analyse the intranuclear movement of the particles from the site of transcription towards the nuclear pore complexes and plan to characterise their diffusion within the nucleosol.

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P57: **Analysis of XMAP215-mediated microtubule growth by TIRF microscopy**

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The proper regulation of microtubule dynamics is essential for many cellular processes. One enzyme that plays a critical role in modulating microtubule behavior is the conserved protein XMAP215, a potent microtubule stabilizer that functions by increasing the rate of microtubule growth and nucleation. We have developed an *in vitro* assay using total-internal-reflection fluorescence microscopy (TIRF) to investigate the interactions of XMAP215 with both stabilized and dynamic microtubules. We demonstrate with single molecule resolution that XMAP215-GFP diffuses in 1D along the microtubule lattice of stabilized microtubules. We further show that XMAP215 recruits free tubulin from solution and that this XMAP215/tubulin complex also diffuses along the microtubule. These results suggest a model whereby XMAP215 promotes microtubule growth by shuttling tubulin dimers to the plus end of microtubules.
To examine this hypothesis further, we modified our TIRF assay to allow for the visualization of dynamic microtubule growth. We demonstrate that the diffusion of XMAP215 is important for microtubule nucleation and growth. Furthermore, we are able to monitor the localization of single XMAP215-GFP molecules along growing microtubules. Utilizing these approaches, we are currently dissecting the mechanistic role of XMAP215 in regulating microtubule dynamics.

P58: **Dynamics of DNA damage induced nuclear domains after laser-UVA-microirradiation**

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Several proteins involved in DNA double strand breaks (DSBs) repair have been shown to form nuclear domains in human cells. MRE11 and RAD51, proteins involved in DNA repair, are recruited to the site containing DNA damage. However, little is known about the regulation of these higher order nuclear architectures associated with DNA repair in human cells.
To examine the dynamic organization of DNA repair protein complexes after induction of DNA damage, we applied local irradiation of cell nuclei with a focused UV-laser (laser-UV-microirradiation). By use of laser-UVA microirradiation the localization of RAD51 at damaged sites containing DSBs could be demonstrated. The accumulation of RAD51 at microirradiated sites was followed in cells fixed at increasing times after microirradiation. First RAD51 accumulations were visible 5 - 10 minutes after irradiation. In contrast to RAD51, the majority of microirradiated nuclei have accumulations of MRE11 already 5 - 10 minutes after irradiation. This is consistent with a report that nuclear MRE11 foci appear early in the response to ionizing radiation. Topological and chronological relationships between several nuclear domains associated with DNA repair examined by a multicolor immunofluorescence staining we have established will also be discussed.

P59: **PPAR dynamics and interaction in living cells measured by fluorescence correlation spectroscopy**

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Peroxisome Proliferator-Activated Receptors compose a family of 3 nuclear receptors, which act as lipid sensors to modulate gene expression as heterodimers with RXR. PPARs are implicated in major metabolic, inflammatory regulations and important processes controlling cellular fate. Fluorescence correlation spectroscopy (FCS), a technique based on the correlation analysis of fluctuation of the fluorescence intensity, was used here for the first time to assess the mobility of PPARs in the nucleus of the living cells. A very interesting result is that unliganded PPARs have small diffusion coefficients. These data reflect the association of PPARs with other proteins and/or their transient interactions with very slow nuclear components. Interestingly, the mobility of the three PPAR isotypes was reduced upon ligand binding[1]. We generate PPAR mutants impaired either in DNA binding or cofactor recruitment and reveal by FCS that chromatin plays a role, but cofactor recruitment is a major determinant for the slow mobility of PPARs. Importantly, we also demonstrate the usefulness of FCS when studying the mobility of nuclear receptors in living cells.

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P60: **New insights into biliverdin reductase functions using fluorescence correlation spectroscopy**

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Biliverdin Reductase is an enzyme known to catalyse the conversion of biliverdin to bilirubin. We studied the diffusion of EGFP-BVR in living HeLa cells using FCS. Our FCS measurements are showing that BVR is moving fast in the cytoplasm as well as in the nucleus. Using the measured diffusion coefficient of EGFP in the same cells as a viscosity standard, we can estimate the size of BVR to be around 160 kDa in the cytoplasm and 220 kDa in the nucleus. This shows that BVR is roaming the cellular environment probably as a EGFP-BVR dimer, without specific interactions. In the presence of heme the mobility of BVR does not change in the cytoplasm, but is slowed down in the nucleus. This may reflect the interaction of BVR with other proteins and/or with very slow nuclear components. Assuming that BVR is associated with soluble factors only, the estimated size in the nucleus in the presence of heme is very important (3 MDa) and it reflects the association with slow nuclear components, such as chromatin. Furthermore, we constructed BVR mutants and study their mobility by FCS. Our study is showing that BVR is associating with chromatin in presence of heme.

P61: **Inhibition of cellular proliferation and modulation of muscle contractility by cell permeable TAT-peptide fusions**

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A basic amino acid peptide from the HIV-1 transactivator of transcription protein (TAT) has been shown to shuttle a wide variety of cargoes into living cells [1,2]. This cell penetrating ability opens up novel non-invasive ways of introducing labels into living cells. We have first tested the cellular uptake mechanism by multidimensional time-lapse confocal microscopy and found that the uptake mechanism depends on the cargoes interconnected to the transduction domain TAT. Whereas fluorescent versions of globular TAT fusion proteins are taken up largely into cytoplasmic vesicles by an endocytic process, small peptides fused to TAT enter the cell in a rapid manner that is dependent on membrane potential and exhibit overall intracellular mobility [3]. Next we assessed whether TAT containing peptides could exert biological effects. We first assayed interference with cellular proliferation by using cell permeable versions of peptides derived from proteins which interact with proliferating cellular nuclear antigen (PCNA). These peptides caused proliferation arrest in myoblast cells as assayed by a novel live cell cycle progression assay. Next we investigated whether we could use the cell penetrating peptide strategy to label and interfere with other cellular functions such as muscle contractility. TAT fused to an N-terminal peptide derived from ventricular light chain 1 protein was applied to primary cultures of adult cardiomyocytes. The fusion peptide was rapidly internalized and specifically labeled sarcomeric structures [4]. Furthermore it increased the intrinsic contractility of cardiomyocytes without changing the intracellular Ca^{2+}-levels. In summary, cell permeable peptides are important tools to label cellular structures in living cells and to interfere with cellular functions, even in primary cells and at over 90% efficiency.

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P62: **The potential role of PML NBs in the regulation of MHC gene expression**

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Promyelocytic leukaemia nuclear bodies (PML NBs) are small nuclear substructures involved in the regulation of gene expression, chromatin dynamics, protein modification, apoptosis, p53 function, senescence, DNA repair, the interferon response, and viral infection. Recently it was shown that PML NBs associate specifically with the histocompatibility complex (MHC) gene cluster in 42 % interphase nuclei of MRC-5 primary fibroblasts.
Here we have analyzed the spatial association of PML NBs and the MHC gene cluster during gamma interferon (γ-IFN) response. In contrast to the published results we observed only 2 % of colocalization between MHC and PML in MRC-5 cells. However, colocalization increased to 10 % in the presence of γ-IFN.
This observation suggested that PML NBs might function as transient storage, assembly or modification sites of proteins involved in the transcription regulation of MHC genes. We therefore tested if such factors would transiently localize to PML NBs during γ-IFN stimulation. To this end we could show that the MHC class II regulators CIITA, RFX-5, and RFX-AP are not enriched at PML NBs. These observations demonstrate that, although spatially linked to MHC genes in an γ-IFN-dependent manner, PML NBs do not serve as sites for accumulation of these specific MHC class II transcription factors.

P63: **Computational imaging PSFs under various conditions in the confocal and the 4Pi microscope**

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To interpret finest details of a biological image correctly, it is an important part to know how the PSF, the answer of the microscope to a point like source, looks like. But even for doing FCS to get mobility parameters and chemical kinetics of certain labelled molecules, it is necessary, to know the observation volume, derivable from the PSF.

Summing up elementarily each possible photon in the microscope to a focus was the way to get the PSFs. From the spherical wave fronts of the objective(s), in a constant angular distance (down to 2°) point like light sources were assumed to interfere in the focal region. Beginning from the optical axis, the spherical wave front created by the objective(s) was divided into circles of growing radius in steps of e.g. 2°, and this circles were divided in discrete sources with the same distance mentioned above of 2°. Here 2 summations had to be performed: over all points of the circle and over all circles. While the axial intensity was the largest, the intensity to the border decreased with cos(n x 2°), and for linear polarised light the bending of the laser beam to the focus changed the direction of the linear polarisation. This directional change was found by a set of rotations for each point and then summed up in the focus. For unpolarised or circular polarised light the computations became easier and faster, because the rotation of the polarisation needed only to be computed for one point, which was automatically the sum over the whole ring divided by the amount of light sources. The calculations led to an amplitude distribution that had to be squared to get the intensity distribution of the illumination.

In fewer words, of importance was, to take the photons from everywhere in the right proportion, phase and polarization, indicated by various microscopic setups.

The detection PSF was the same as the illumination PSF with the difference, that it was convoluted by the pinhole. Finally the observation PSF was a product of the illumination and detection PSF. For the 2-photon case it was only necessary to square the illumination PSF and than to multiply with the detection PSF.

Different series of PSFs with variation of 1 Parameter each will be presented.

P64: **Applicability of fluorescent protein variants for live cell microscopy and photodynamic studies**

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Since the cloning of fluorescent proteins a decade ago, they have revolutionized the microscopic analysis of cells by enabling live cell microscopy. The first autofluorescent protein GFP and its spectral variants are widely used in cells and even organisms. Subsequently many red-shifted autofluorescent proteins were identified and their DNAs isolated. With the development of better and faster microscopes, dual and triple colour live cell imaging has become feasible. Recently, new varieties of the monomeric red fluorescent protein have been developed [1,2]. We have analyzed three variants of mRFP1 - mCherry, mOrange and mPlum - for their usage in multicolour imaging, FRAP and FRET.
Fusion proteins of mCherry, mOrange and mPlum with PCNA, a very well characterized protein, were established. With transient transfections, we analysed the influence of the fluorescent proteins on the correct localisation of PCNA. In live cells we determined the emission spectra and the photobleaching properties using various laser lines. We are generating stable cell lines using a site-specific recombination system and are testing the usefulness of these fluorescent proteins in long-term live cell imaging. Furthermore, we can analyze their applicability for FRET studies in living cells.
With this study we aim to provide a guideline for the use of red fluorescent protein variants in live cell microscopy.

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P65: **Intracellular interaction between syntaxin and Munc 18-1 revealed by fluorescence resonance energy transfer**

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Neurosecretion is catalyzed by assembly of a SNARE-complex composed of SNAP-25, synaptobrevin and syntaxin. Munc 18-1 is known to bind to syntaxin in-vitro. This interaction prevents assembly of the SNARE-complex, but might also affect intracellular targeting of the proteins.
We have fused syntaxin and Munc 18 to the yellow- (YFP) or cyan-fluorescence-protein (CFP) and expressed the constructs in CHO- and MDCK-cells. We have studied their localization with confocal microscopy and a possible protein-protein-interaction with fluorescence-resonance energy transfer (FRET). YFP-syntaxin localizes to intracellular membranes. CFP-Munc 18 is present in the cytoplasm as expected for a protein lacking membrane targeting domains. However, Munc 18 is redirected to internal membranes when syntaxin is coexpressed, but only limited transport of the proteins to the plasma membrane was observed. An interaction between Munc 18 and syntaxin could be demonstrated by FRET using two methods, sensitized acceptor fluorescence and acceptor photobleaching. A mutation in syntaxin (L165A, E166A), which is known to inhibit binding to Munc 18 in-vitro, prevents colocalization of the proteins and also the FRET signal. Thus, a protein-protein interaction between Munc 18 and syntaxin occurs on intracellular membranes, which is required but not sufficient for quantitative transport of both proteins to the plasma membrane.

P66: **Fluorescence Lifetime Imaging Nanoscopy (FLIN)**

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FLIN, as recently introduced in *SingleMotorFLIN* (NMP4-CT-2005-013880), provides groundbreaking tools for the study of single molecules (SM) and single molecular motors (SMM), as well as a broad array of phenomena in NanoWorld. Classical limitations in SM/SMM studies, such as resolution, short observation times, and photo-dynamic reactions are overcome by minimal-invasive picosecond FLIN. FLIN is the extension of the extremely successful fluorescence lifetime imaging microscopy (FLIM) into the nano-domain, with down to 10 nm space-resolution. FLIN results from the combination of nanoscopy (such as multi-colour, wide-field, point-spread- function (PSF) modelling microscopy) with novel ultrasensitive, non-scanning imaging detectors, based on time- and space-correlated single photon counting (TSCSPC) that allows ultra-low excitation levels. This results, for example, in long-period (>1 hour), minimal-invasive observation of living cells and SM/SMM, without any cell damage or irreversible bleaching. Minimal-invasive FLIN with global PSF-modelling allows observation of point-source movement at 1-nm accuracy and distance determination at the 10-nm level, while simultaneously acquiring multi-exponential pico/nanosecond fluorescence dynamics. FLIN opens a wide avenue of novel applications, such as SMM-tracking, FRET-verification, dual-polarisation tracking, and super-background-free 2-photon TIRF-FLIN. *SingleMotorFLIN* will examine the behaviour of four types of SMM and their dependence on energy-input. Enhanced basic understanding of biological and artificial machines/motors will lead to advanced models and proceed one day to artificial systems, revolutionising the interface of biological and non-biological worlds. Since biological SMM are involved in many human disorders, the novel FLIN method will help to show how these motors operate and how they break down in disease.

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P67: **Centromere assembly through highly immobile and some mobile foundation kinetochore proteins**

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We have analysed the dynamic behaviour of the foundation kinetochore proteins CENP-A, CENP-B, CENP-C, CENP-I, and hMis12 in living human cells using fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS). In interphase cells, CENP-A, CENP-B, CENP-C, and CENP-I are stable components of the kinetochore over hours. In contrast, hMis12 rapidly and completely exchanges within seconds. During mitosis CENP-A, CENP-C, and CENP-I remain stably associated with kinetochores, while CENP-B becomes mobile and, strikingly, hMis12 becomes completely immobilized at kinetochores. FCS detected soluble pools with diffusion coefficients between 1 and 2 $\mu m^2 sec^{-1}$. This diffusional behaviour is consistent with transient chromatin interactions of foundation kinetochore proteins outside of centromeric DNA. In contrast to many other subnuclear complexes but similar to nucleosomes, our observations identify the kinetochore as a nuclear DNA/multiprotein assembly which is not determined by a constant flux of exchanging components. However, alterations in the mobility of specific kinetochore proteins appear to be associated with the changing functional properties of centromeres during mitosis.

P68: **Assessing the real size distribution of germinal centers**

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Germinal centers (GC) are structures that form in secondary lymphoid organs like spleen and lymph nodes during an immune response and are the places where affinity maturation of antibodies takes place. By analysis of immunostained tissue sections the life time of GC was ascertained to be about 3 weeks with GC first arising at day 4 p.i., followed by an expansion phase with the mean size of GC reaching its peak at day 10 p.i. and a subsequent gradual decline phase of GC up to day 21 p.i. We record the size of GC in terms of areas [μm^2] in 25 µm murine splenic tissue sections as a function of the time by large-scale confocal imaging of whole spleen sections. Samples were immunostained for B cells (mAb Ki-67), T cells (mAb CD3), FDC network (mAb FDC-M2) and macrophages (mAb CD68). Whereas the variance of the mean size of GC, defined by their B cell population at a given time point is small between several individuals, the variation between GC areas within a single section emerged as being very high leading to a broad distribution of GC areas. The estimation of the real size distribution of GC in the original three-dimensional spleen sample is a Wicksell´s corpuscle problem. To solve this problem we parametrize the detected GC areas by an ellipse fitting algorithm and plot the ratio of the according major and minor radii of the fitted ellipses. In a theoretical approach we assume GC as ellipsoids in the three-dimensional space and perform virtual random sectioning of various different shaped ellipsoids. The distribution of areas and the ratio of major and minor radii derived by virtual random sectioning are then compared to three-dimensional reconstructions of individual GC, scanned at high magnification over several sections.

P69: **Assembly of active zone precursor vesicles: obligatory trafficking of presynaptic cytomatrix proteins via a trans-Golgi compartment**

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Neurotransmitter release from presynaptic nerve terminals is restriced to active zones, which are characterized by a complex of cytoplasmic scaffolding proteins termed the presynaptic cytomatrix. To analyze the biogenesis of this molecular complex, we have asked how the cytomatrix molecules Bassoon and Piccolo are delivered to synapses. Although these proteins may be transported via vesicles, little is kown about the molecular determinants and the importance of a vesicular pathway. We find that both proteins co-localize at a subcompartment of the trans-Golgi network in cultured neurons. Impairing vesicle exit from the Golgi complex, either using Brefelding A, recombinant proteins or a low temperature block, prevents transport of Bassoon out of the soma. Deleting a Golgi-binding region of Bassoon impairs subcellular targeting of recombinant Bassoon. Overexpressing this region to specifically block Golgi-binding of the endogenous protein reduces the concentration of Bassoon at synapses. We are using live imaging of Neuroligin-induced active zone formation to further analyze the trafficking of cytomatrix precursors between the Golgi-apparatus and synaptic terminals. Our date suggest that during synaptogenesis a primordial cytomatrix assembles in a trans-Golgi compartment and that transport via Golgi-derived vesicles is essential for delivery of cytomatrix proteins to the synapse.

P70: **Analysis of HIF-1a and ARNT interaction and their mobility in living cells**

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Cellular oxygen sensing and coordination of an adequate response towards hypoxia are essential for surviving of organisms exposed to variations in oxygen supply. The transcription factors complex hypoxia inducible factor-1 (HIF-1) as the master regulator of cellular O_2 homeostasis is formed by an O_2-sensitive α- and a constitutive β-subunit (ARNT). Regulation of the assembly and the activity of this complex are key events in cellular O_2-sensing. Confocal microscopy provides techniques like fluorescence resonance energy transfer (FRET) to study protein-protein interactions involved in this sensing pathway in living cells and to understand the dynamics of the interaction of these molecules. Additionally the mobility and diffusion characteristics of HIF-1a and ARNT inside living cells can be investigated by the fluorescence recovery after photobleaching technique (FRAP).
By 2-photon microscopy we have analysed the 3D structure of the HIF-1 complex using fusion proteins of partners of the HIF-1 complex and fluorescent protein assembly of the complex. Our results provide an insight into the protein-protein interaction and protein movement processes inside living cells under hypoxic conditions.

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P71: **Direct observation of sterol transport and distribution in living cells**

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Cholesterol is an important constituent of cellular membranes playing a fundamental role in many biological processes including regulation of membrane permeability, lateral lipid organization, signal transduction and membrane trafficking. Using the natural, intrinsically fluorescent cholesterol analog dehydroergosterol (DHE) sterol transport has been studied in living mammalian cells. By adapting a conventional epifluorescence microscope with specific filter sets DHE's fluorescence emission being maximal in the UV region of the spectrum (370 to 400 nm in membranes) has been collected[1]. Using detailed pulse-chase protocols it is shown that DHE moves by vesicular and non-vesicular modes in cells, and that recycling endosomes represent a major sterol pool in non-polarized and polarized cell types[2, 3]. By fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP) and time-lapse imaging DHE transport kinetics were determined. It was found that DHE can exchange between cellular compartments within 1-2 min even in the absence of metabolic energy[2, 3]. By correcting for DHE bleaching improved image resolution was obtained and DHE could be resolved in microvilli of the canalicular membrane of polarized hepatic cells[4]. By incorporating DHE into fluorescence tagged HDL it was found that non-esterified sterol is rapidly released at the basolateral cell surface and shuttled to the canalicular membrane by non-vesicular transport[2, 5]. A key event during atherogenesis is the conversion of blood-derived macrophages into foam cells. By developing specific image analysis protocols it was found that DHE release from the plasma membrane is accelerated in model foam cells compared to normal macrophages[3]. We relate this observation to the known threshold phenomenon of cholesterol dependent cholesterol esterification in foam cells during atherogenesis.

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P72: **Developoment of the FRET-based method for imaging enzyme activity in living cells**

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Subcellular partitioning and regulation of enzyme specific activities evade studies by conventional activity assays. Though indispensable in determination of substrate specificity and catalytic mechanisms, they do not faithfully represent enzyme operation in living cells. We have developed a novel method for imaging enzyme-substrate (ES) intermediates in living cells by monitoring FRET between donor fluorophore-tagged enzyme and a synthetic acceptor-labeled substrate, which enables specific visualization of the reporter-occupied active sites and highlights localized dynamics and regulation of enzyme activity in cells. We used this approach to spatially resolve operation of PTP1B phosphotyrosine phosphatase in living cells.

The approach was first tested *in vitro* using purified, bacterially expressed EGFP-tagged catalytic domain of PTP1B (residues 1-321) D181A mutant and the lissamine-labeled synthetic substrate. Formation of ES complex results in FRET observed as EGFP quenching, increased lissamine emission and decreased EGFP lifetime. Consistent with PTP1B catalytic mechanism, FRET is attenuated by thiol-reactive compounds or competition with unlabeled substrate. Incubation of the reporter substrate with the wild type PTP1B resulted in transient FRET signal with kinetic parameters typical for PTP1B (K_M 2.21 μM; k_{cat} 66.6 s^{-1}).

In cells, fraction of ES complex over total concentration of donor-tagged PTP1B can be determined quantitatively using fluorescence lifetime imaging microscopy (FLIM). FRET in the ES complex is detected as a fraction of donor-tagged enzyme with a decreased fluorescence lifetime that can be distinguished from the free enzyme population with higher lifetime using global analysis. Imaging ES complex of EGFP-PTP1B^{WT} with the reporter substrate in live cells, we demonstrate the establishment of the steady-state regime in the kinase/phosphatase reaction cycle and observe distinct steady-state substrate fluxes through the PTP1B in subcellular compartments. By analyzing a cellular map of the apparent K_M for PTP1B, reconstructed from images of the ES intermediate, we demonstrate that inside cells PTP1B exists as spatially separated kinetically distinct subpopulations and present evidence for functional significance of these subpopulations inside cells.

The proposed method of imaging enzyme-substrate intermediate in living cells may be used in studies of steady-state flux of metabolites through metabolic pathways and signaling cascades, functioning and regulation of enzyme activities and localization of the available enzyme active sites in a complex cellular environment.

P73: **Statistical detection of fluorescent rods in noisy 2D images with super-resolution**

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We describe a new method[0] designed to detect fluorescent rods from noisy 2D microscopy images. It is motivated by the desire to study the dynamics of bacteria such as *Shigella*[2]. The methodology is adapted from a super-resolution spot detection algorithm[3,1] and is based on a parametric model of the rods and the microscope point spread function. The algorithm consists of two parts: 1) a pre-detection step, based on thresholding a score computed from the product of the mean curvature and the local intensity of the filtered image, 2) an iterative procedure, where a mixture model of blurred segments is fitted to the image, and segments are first removed, then added according to whether the agreement of the model with the image changes significantly or not. The hypothesis testing approach used throughout the algorithm allows an explicit control over the probability of false alarm (falsely detecting a rod in noise). We experimentally estimate the performance of our algorithm on simulated images for typical imaging conditions. We find that the algorithm can reliably detect isolated rods at signal to noise ratios as low as 2.5 and localize them with an accuracy of 20 nm or better. We also show that it can distinguish rods separated by distances below the Rayleigh resolution limit. Finally, we illustrate the algorithm's ability to identify and separate overlapping bacteria on a real image (see figure below). We are now extending this work to 3D+time imaging data and better geometrical model of fluorescent bacteria.

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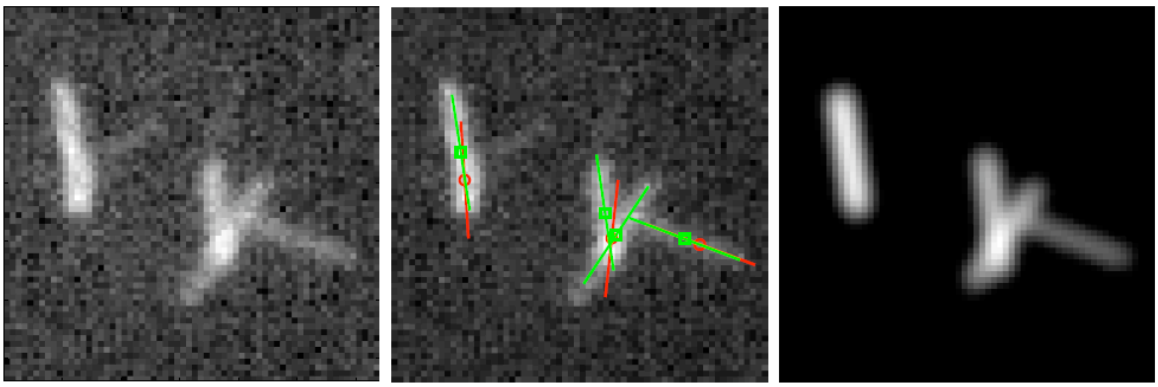

P74: **Building high resolution maps of gene positions in yeast nuclei by automated 3D image analysis**

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The localization of specific genes inside the nuclear volume is both non random and linked to transcriptional activity[1]. Intranuclear localization thus provides an important gene regulation mechanism, but its generality and the underlying physical mechanisms are still poorly understood. To explore this further, we need more systematic and detailed quantitative descriptions of 3D gene locations with respect to important spatial landmarks such as the nuclear envelope, the nucleolus and the spindle pole body. Because genes are also known to undergo stochastic motions[2,3], a statistical analysis on large amounts of data is essential. Here, we will report on our efforts to build high resolution maps of spatial gene distributions by automated analysis of thousands of 3D images of fluorescently labeled genes in yeast nuclei. Yeast allows easy manipulation of the transcriptional status, but the cell diameter of only 2-3 micrometers means that images of intranuclear structures are strongly blurred by diffraction. Nevertheless, we will show that an image processing approach based on statistical detection and estimation can locate genes relative to other nuclear structures with a precision higher than the diffraction limit. Geometric alignement of the locations extracted from each nucleus then enable us to condense the information from whole populations into meaningful probability density maps (see example below). Such maps provide a visualization of where genes are located relative to the nucleolus and other nuclear landmarks. We expect that this approach, which allows a significantly deeper look into the nuclear architecture, will be of great value to the study of gene positioning and function.

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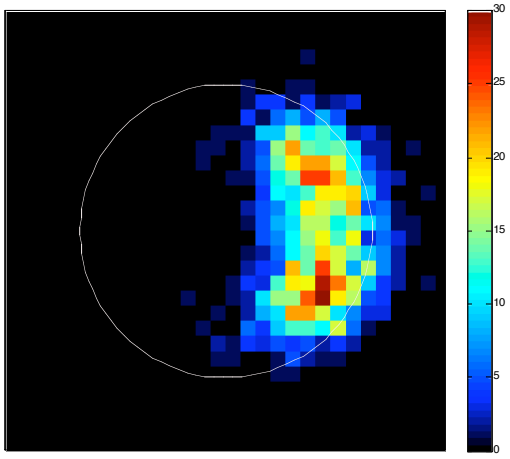

P75: **SMI – nanosizing of COMBO – FISH labels complementary to ABL sequences in human blood cell nuclei**

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COMBinatorial Oligo Fluorescence *In Situ* Hybridization (COMBO-FISH) [1] offers an approach to label double strand DNA in cell nuclei by means of microscopically co-localizing, fluorescence labeled oligonucleotides. From the human genome data base a set of 31 homopyrimidine oligo-stretches was computer designed and synthesized that was complementary to sequences in the ABL region of 225 kb. Some of the oligonucleotides had additional binding sites somewhere else in the genome but clusters with more than 6 probes were excluded [2]. Thus the probe set exclusively co-localized within a chromatin domain corresponding to the diffraction limited point volume of a high numerical aperture lens. By means of Spatially Modulated Illumination (SMI) microscopy the sizes of these fluorescent chromatin domains carrying 62 OregonGreen dye molecules on 31 probes were measured [3] in human lymphocytes and in blood cells of CML patients before and after bone marrow transplantation fixed on glass slides. For more than 250 cell nuclei image stacks of about 200 slices were recorded and evaluated. The sizes of the labeled chromatin domains ranged from 50 nm to 200 nm in diameter. The mean values ranged between 113 nm (CML patient in the remission state) to 152 nm (lymphocyte specimen). A statistical evaluation of the cumulative frequency histograms of the chromatin domain sizes using the Kolmogorov-Smirnov test revealed significant differences between the blood cells of the CML patients and the lymphocyte specimens. In addition, significant differences were obtained between CML patients before and after bone marrow transplantation. So far only a very low number of patients have been evaluated. However, the data indicate the usefulness of COMBO-FISH and SMI-microscopy for the estimation of chromatin compaction in breakpoint regions like ABL or BCR.

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P 76: **Quantum dots in multi-colour FISH**

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Quantum dots (Qdots) are semiconductor nanocrystals (CdSe or CdTe). Qdots are photo-stable, show bright fluorescence with narrow, symmetric emission spectra and are available in multiple resolvable colours. Their use in biological applications, for example in FISH experiments, has been investigated for several years, however only recently stable and water soluble Qdot conjugates have become commercially available. Interestingly, all Qdots can be excited simultaneously using a single excitation wavelength of 425nm. Because of their large Stoke`s shift ranging between 100nm and over 250nm they can be potentially used to expand the number of fluors to be simultaneously used in multiplex FISH experiments when combined with organic fluors.

We therefore established a FISH protocol, which allows the simultaneous visualisation of DNA probes labelled with Qdots and with conventional fluors. Using a SP2 laser scanning confocal microscope (Leica Microsystems) with accusto-optical beam splitters for visualisation, we tested various combinations of hapten-labelled probes detected with Streptavidin-Qdot 525, anti-Digoxigenin-Qdot 605 and anti-Dinitrophenol Qdot 655, respectively, and FITC-dUTP, Cy3-dUTP and TexasRed-dUTP labeled probes. Using this microscopic setup, spectral separation of these six fluors was satisfying. In our hands, however, the overall performance of the Qdot conjugates tested varied considerably. Streptavidin-Qdot 525 showed best results, followed by anti-Dinitrophenol Qdot 655 and anti-Digoxigenin-Qdot 605, indicating that the differences observed are possibly not attributed to increased Qdot size, which is positively correlated with emission wavelength. In addition, we are currently evaluating whether Qdots are suitable for high resolution imaging of FISH probes using 2-photon excitation 4Pi microscopy, which holds promise to push the limits of resolution of light microscopy to less than 100 nanometers, but requires the use of extremely photo-stable fluors.

P 77: **Active leukocyte crawling in microvessels assessed by digital time-lapse intravital microscopy**

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The ability of active movement is an important feature of leukocytes. Conventional (VHS) video microscopy utilizing off-line analysis of analog video signals is suitable for the same purpose if it is processed by consecutive digital capturing. However, the use of this technique for comprehensive analyses of microcirculation is very difficult, because it is time-consuming and admittedly requires a lot of patience by the observer. Here, we used a hybrid technique which combines intravital microscopy and digital time-lapse video microscopy to investigate the physiology and molecular mechanisms of intravascular leukocyte movement.
This technology offers several advantages compared to conventional microscopic observations. First, the time-lapse video recording allows to compress the observation interval in a free-defined relation. It allows for continuous observation of all changes in leukocytes including attachment, polarisation and migration. Second, the method facilitates to follow the intravascular, transendothelial and extravascular movement of every single leukocyte with optional acceleration. Finally, this technology allows the exact assessment of several important parameters such as the direction, distance, time and velocity of leukocyte movement. As shown by the baseline measurement, the technique provides a stable status of leukocyte locomotion in the intra- and extravascular space.
Intravital microscopy of mesenteric venules was performed in male Wistar-rats using digital video recording and time-lapse image compression. The leukocyte movement and extravasation were analysed after local application of TNF-α, after blockade of endothelial (anti-ICAM-1 antibody) and leukocyte (anti-CD18 antibody) adhesion molecules. Additionally, the migratory activity of isolated leukocytes in collagen gel was analysed and compared with their intravascular locomotion.
Adherent leukocytes showed an active intraluminal crawling along the endothelial lining. Most permanent stickers (84 ± 13%) crawled actively on the intraluminal site of venules. Baseline measurement of leukocyte crawling velocity yielded an average 9.0 ± 1.8 µm/min which was not significantly different from crawling velocity of extravascular leukocytes (8.9 ± 4.5 µm/min). The maximum distance of leukocyte crawling observed was 150 µm. The maximum time of crawling was 15 min. Intraluminal crawlers travelled over a mean distance of 35 ± 17 µm with the average duration of 5.4 ± 1.4 min. Under unstimulated conditions, almost all crawling leukocytes detached from the endothelium and did not migrate through the vascular wall. TNF-α induced a significant increase of leukocyte extravasation. Anti-ICAM-1 and anti-CD18 antibodies significantly reduced leukocyte crawling. The proportion of isolated migrating leukocytes in collagen gel (87 ± 6%) was not significantly different from the percentage of intravascular crawling leukocytes in vivo.
The method of digital time-lapse intravital microscopy represents an advantageous technology for the investigation of intravascular, transendothelial and extravascular migration of leukocytes. Using this technology, we showed that leukocyte- -endothelial-interactions are an active and dynamic process. This process involves long-time (several minutes) crawling of leukocytes along the endothelium and, finally, detachment from the endothelium. Intravascular leukocyte crawling reflects the migratory potential of circulating

leukocytes and strongly depends on the expression of adhesion molecules. For extravasation, an additional pro-inflammatory stimulus is required.

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Junger Mann mit Schal (Portrait Max Delbrück), Jeanne Mammen, um 1935-40