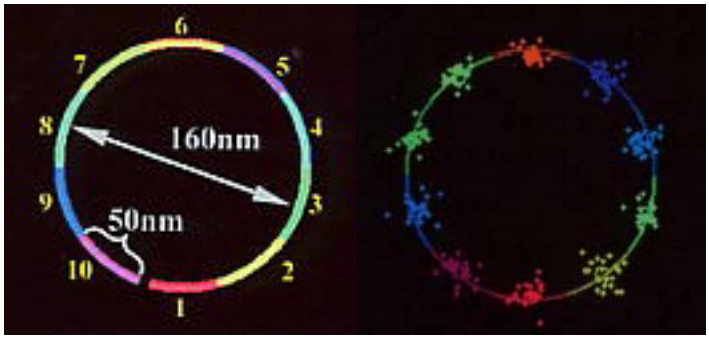
BMM

DFG
SPP 1128

Kirchhoff-Institute of Physics
Bioquant
University Heidelberg

October 25, 2007

Workshop:
Spectrally Assigned Localisation Microscopy (SALM) of Cellular Nanostructures



H. Münch/KIP

Programme

Kirchhoff-Institute (Kleiner Hörsaal)

8.45 h	Welcome, Registration
9.15 h	Opening, Principles of Localisation Microscopy Christoph Cremer, Heidelberg
10.00 h	Nanoscale Intracellular Imaging by Fluorescence Photoactivation Localization Microscopy Sam Hess, Orono
10.45 h	Coffee Break (Posters)

Chair: Michael Hausmann

11.15 h	Analysis of the human kinetochore Stephan Diekmann, Jena
11.45 h	Modelling of Cellular Nanostructures Dieter Heermann, Heidelberg
12.15 h	Far-Field Nanoscopy with Optical Sectioning Capabilities by Fast Single Molecule Photo-Switching Jonas Fölling, Hell Group Göttingen
12.45 h	Lunch

Chair: Sam Hess

14.00 h	Molecular Angström Optics: A dynamic view in BioSoft matter Claus Seidel, Düsseldorf
14.30 h	Advanced Fluorescent Proteins for Localization of Cellular Nanostructures Uli Nienhaus, Ulm
15.00 h	Focussed labelling of genome nanostructures by COMBO-FISH Michael Hausmann, Heidelberg
15.30 h	Coffee Break (Posters)

16.00 h	Novel Approaches in single molecule spectroscopy for investigations of living cells Dirk-Peter Herten, Heidelberg
16.30 h	Statistical aging of eGFP fluorescence photobleaching dynamics V. Shynkar, Paris
16.50	Localization fluorescence Microscopy using Quantom Dot blinking R. Heintzmann, London

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17.30 h	Open End Discussion: Exploration –Collaboration Potential (Please register separately for this part.)

Nanoscale Intracellular Imaging by Fluorescence Photoactivation Localization Microscopy

Samuel T. Hess, Travis J. Gould, Manasa V. Gudheti, and Joshua Zimmerberg

Biological structures span many orders of magnitude in size, but far-field visible light microscopy suffers from limited resolution. A new method for fluorescence imaging has been developed that can obtain spatial distributions of large numbers of fluorescent molecules on length scales shorter than the classical diffraction limit. Fluorescence photoactivation localization microscopy (FPALM) analyzes thousands of single fluorophores per acquisition, localizing small numbers of them at a time, at low excitation intensity. In order to control the number of visible fluorophores in the field of view and ensure that optically active molecules are separated by much more than the width of the point spread function (PSF), photoactivatable fluorescent molecules are used, in this case the photoactivatable green fluorescent protein (PA-GFP). Non-fluorescent inactive PA-GFP molecules are first activated by 405 nm laser illumination and then imaged by CCD camera under illumination by an Ar+ ion laser, which also eventually photobleaches those active molecules, removing them from the field of view. PA-GFP-tagged influenza hemagglutinin (HA) expressed in live and fixed fibroblasts is imaged by FPALM with estimated localization-based resolution of ~30-40 nm.

Analysis of the human kinetochore

Daniela Hellwig, Peter Hemmerich, Stephan Diekmann
FLI, Beutenbergstr. 11, 07745 Jena

We analyse structure and function of the human kinetochore. We recombinantly tagged and labelled the known human centromere proteins including the NAC, CAD, HEC1 and hMis12 complexes. We studied the interaction of these proteins *in vivo* and *in vitro*.
While most kinetochore proteins have been identified by now, nothing is known about the assembly dynamics of this chromosomal substructure. Using quantitative bleaching and fluorescence correlation spectroscopic imaging, we have assessed the incorporation and exchange rates of six inner kinetochore proteins during all phases of the cell cycle in living human cells. We demonstrated that CENP-A and CENP-I are loaded into kinetochores exclusively in G1 and S phase, respectively. CENP-B, CENP-C, CENP-H and hMis12 exhibit distinct and cell cycle-specific chromatin binding stabilities with residence times ranging between seconds to hours. CENP-C is immobilized at kinetochores specifically at mid to late S phase indicating the requirement for stable CENP-C binding to the inner kinetochore during centromere replication. At mitosis all inner kinetochore proteins become completely immobilized at centromeres. Outside centromeres, inner kinetochore proteins are highly mobile. This behaviour is consistent with a model in which these kinetochore proteins scan the nucleus by a binding-diffusion mechanism in search for their high-affinity binding sites at centromeres.
We tagged the inner kinetochore proteins CENP-A, -B, -C, -H and –I as well as the histones H2A, H3, H4 and H1 by Cerulean and EYFP, many of them at both termini, and analysed the energy transfer between them in human living cells. We found CENP-A and CENP-B in close vicinity to one another *in vivo*. In further experiments, we observed dimerisation of CENP-B and CENP-C with themselves as well as an interaction with one another. By energy transfer we obtained data indicating that the linker histone H1.0 is present in centromeric chromatin *in vivo*.

Far-Field Nanoscopy with Optical Sectioning Capabilities by Fast Single Molecule Photo-Switching

Jonas Fölling

"The resolution of classical far-field fluorescence microscopy is limited by diffraction. Different methods such as RESOLFT- and PALM-type microscopy have evolved over the past years which truly overcame this physical barrier, utilizing the intrinsic "on" and "off" states of the fluorescent marker not only for signal acquisition but also to ensure that the signal stems only from a sub-diffraction region. PALM-type microscopes reconstruct objects at the nanoscale by subsequently activating and deactivating the fluorescence of single isolated molecules at random positions and determining their positions with sub-diffraction accuracy. Repeating this process multiple times yields accurate spatial information about the stained object. Here, we report a new photochromic rhodamine derivative for PALM-type microscopy. Its large brightness allows for a very good localization precision, leading to high image resolution. The fast switching of the molecules in combination with an asynchronous (PALMIRA) recording scheme lead to short image acquisition times. Additionally, the virtually infinite contrast ratio between on- and off-state and the very low fraction of thermally activated dye molecules enable us to minimize background contribution and to use conventional far-field microscopy. This fact and the ability to activate our rhodamine dyes using a two-photon process make it possible to image thick samples using noninvasive optical sectioning and extend PALM to three dimensional imaging."

Advanced Fluorescent Proteins for Localization of Cellular Nanostructures

Gerd Ulrich Nienhaus

Institute of Biophysics, University of Ulm, 89069 Ulm, Germany, and

Department of Physics, University of Illinois at Urbana-Champaign,

Urbana, IL 61801, USA.

Fluorescence microscopy has taken a prominent role in life science research due to its ability to provide quantitative, spatially and temporally resolved information on biological processes both *in vitro* and *in vivo*. Recent years have witnessed substantial advancements in instrumental performance. A variety of sophisticated microscope designs have been developed, and some of them even afford spatial resolutions well below the Abbe limit. Fluorescent markers are of key importance in this area, especially genetically encoded fluorescent proteins (FPs) of the green fluorescent protein (GFP) family, which can be employed as minimally perturbing endogenous probes for live-cell imaging. By using genetic engineering of naturally occurring fluorescent proteins, novel markers have been developed with improved and even entirely novel properties, which may considerably widen the range of possible applications.

In recent years, we have characterized a large number of anthozoan FPs with interesting properties, including red fluorescent and green-to-red photoactivatable variants. These have been cloned and subjected to further optimization using genetic engineering [1-3]. We will discuss recent biophysical characterizations of photoactivatable FPs using x-ray crystallography, bulk and single-molecule spectroscopy, and we will also present applications of EosFP in 4Pi confocal microscopy with two-photon excitation [4].

[1] Wiedenmann, J., Schenk, A., Röcker, C., Girod, A., Spindler, K.-D. & Nienhaus, G. U., A Far-Red Fluorescent Protein with Fast Maturation and Reduced Oligomerization Tendency from *Entacmaea quadricolor* (Anthozoa, Actinaria), Proc. Natl. Acad. Sci. USA 99 (2002) 11646.

[2] Wiedenmann, J., Ivanchenko, S., Oswald, F., Schmitt, F., Röcker, C., Salih, A., Spindler, K.-D. & Nienhaus, G. U., EosFP, A Fluorescent Marker Protein with UV-Inducible Green-to-Red Fluorescence Conversion, Proc. Natl. Acad. Sci. USA 101 (2004) 15905.

[3] Nienhaus, K., Nienhaus, G. U., Wiedenmann, J., & Nar, H., Structural Basis for Photo-Induced Protein Cleavage and Green-to-Red Conversion of Fluorescent Protein EosFP, Proc. Natl. Acad. Sci. USA 102 (2005) 9156.

[4] Ivanchenko, S., Glaschick, S., Röcker, C., Oswald, F., Wiedenmann, J., & Nienhaus, G. U., Two-photon Excitation and Photoconversion of EosFP in Dual-color 4Pi Confocal Microscopy, Biophys. J. 92 (2007) 4451.

Statistical aging of eGFP fluorescence photobleaching dynamics

V. Shynkar[a], F. Amblard[a]

[a]*Laboratory "Physics of the cytoskeleton", UMR 168, Institut Curie, 75248 Paris, France*

The large family of fluorescent proteins became extremely popular tools during the past decade, but the photophysics of those proteins is not completely understood, especially from the point of view of its bleaching properties. We used one of the GFP variant, eGFP, to dissect the dynamics and statistics of fluorescence bleaching, and its mechanism. Fluorescence measurements of the chromophore bleaching were performed over a wide set of time scales by using photon counting technique. The results of experiments demonstrate the time dependence of bleaching time constant of eGFP and can be modelled by a combination of irreversible bleached state and reversible dark state. The former process obeys Poisson statistics, and the later one follows a Levy distribution. This time dependence of the emission statistics is reminiscent of similar observations in single molecular enzymology experiments. We suggest that time dependence reflects the non-ergodic character of protein configurational dynamics. The results of this investigation will have fundamental implications on the relationship between protein dynamics and fluorescence emission, and practical impact on photobleaching-based methods.

Localization Fluorescence Microscopy Using Quantum Dot Blinking

Keith A. Lidke, Rainer Heintzmann

Blinking of fluorescent quantum dots can be used to discriminate them, which allows their successive localization. One method of separating non-moving blinking objects from a time series of images is by using independent component analysis (ICA). Here we investigate with the help of simulations, how well 5 particles within the radius of one point spread function can be separated and localized and how the precision of object position depends on various imaging parameters. As proposed previously (Lidke et al., Opt. Expr. 13, 2005) the vision is to take the step from localizing to painting an image, which we called "Pointillism". In contrast to methods based on identification of subframes showing only single particles, which practically limits the method to distance measurements between two targets, separation methods such as ICA can separate several overlapping particle images.

(from Lidke & Heintzmann, Proc. IEEE 936-939 (2007))