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Lightoptical Nanoscopy @Molecular Optical Resolution

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A basic goal of biophysics is to quantitatively understand cellular function as the consequence of the fundamental laws of Physics governing a system of extreme complexity. For this, detailed spatial information about the nanostructures involved is necessary. For example, the nanostructure of the chromatin (DNA + specific proteins) is of essential importance for the way the activities of the thousands of genes in the cell nucleus are regulated[1]. A serious problem to analyse such cellular nanostructures by far field light microscopy has been the conventional optical resolution restricted to about 200 nm laterally (ca. ½ of the wavelength used) and 600 nm axially ("Abbe/Rayleigh-limit"). For about a hundred years, the Abbe/Rayleigh limit (1873/1896) based on the nature of electromagnetic waves was thought to pose an absolute, unsurmountable limit for any attempts to lightoptically analyse the nanostructure of intact cells, the basic units of life. Although imaging methods based on ionizing radiation such as electron microscopy have partly filled this gap of resolution, improved lightoptical approaches remained of utmost importance.

A first successful attempt to overcome this barrier at least in one direction was confocal laser scanning fluorescence 4Pi microscopy[2-4]. In 2005, such a "superresolution" 4Pi microscope manufactured by the Leica Company has been established at the Kirchhoff-Institute and since than has been used[5] for cellular bioimaging at the 100 nm axial optical resolution level (about 6 times beyond the conventional limit). Simultaneously we developed various other laseroptical methods of far field fluorescence microscopy to realize

a spatial analysis of ‘biomolecular machines’ (highly complex intracellular structures composed of macromolecules such as proteins and DNA) far beyond the magical Abbe/Rayleigh-limit. For example, we have established methods of structured laser illumination with a lateral optical resolution substantially below the Abbe/Rayleigh limit[6]. A particular focus was Spatially Modulated Illumination microscopy[7,8] which made it possible to determine with high precision the size of biomolecular machines in the cell interior down to few tens of nanometer, corresponding to about 1/15 of the exciting wavelength used. Analyzing thousands of such ‘machines’, we succeeded to measure average structural changes in the 1 nm range (for comparison: ribosomes needed for the synthesis of proteins have a diameter of few tens of nm).

In another approach, we focused on the further development of “Spectral Precision Distance/Position Determination Microscopy” (SPDM). SPDM is a far field fluorescence technique based on labelling ‘point like’ objects (e.g. single molecules) with different spectral signatures (e.g. light induced reversible or irreversible conformation changes which can be optically distinguished[2], changes in the fluorescence emission spectrum, life time etc.[9 - 12]), spectrally selective registration and high precision localization monitoring by far field fluorescence microscopy. (Also called “Spectrally Assigned Localization Microscopy”, SALM). After the development of the concept of SPDM in the 1990s[9-11], we performed first ‘proof-of-principle’ experiments at the end of the decade in collaboration with our partners at LMU Munich, Tel Hashomer Hospital (Israel) and the National Cancer Institute (Bethesda/USA)[13], realizing a three-dimensional optical resolution (smallest distance detected between ‘pointlike’ fluorescent sources) of about 50 nm (ca. 1/10th of the exciting wavelength). In these experiments, however, only a few spectral signatures based on differences in the fluorescence emission spectrum were used.

During the last two years, we extended the SPDM approach from few to hundreds of spectral signatures using “physically modifiable fluorochromes”. These SPDM$_{Phymod}$ methods[14 - 17] made it possible for the first time to analyse intracellular nanostructures at single molecule detection (at densities of about 1,000 molecules/µm^2 of the same type) and with molecular optical resolution (down to the 10 nm range), using laser lines in the visible (low energy photon) range; this was achieved with far field techniques (large working

distances) and standard fluorophores, from conventional fluorescent proteins to widely used small fluorochrome molecules. Such superresolved images were obtained even under live cell conditions. In addition, we were able to combine the SPDM$_{Phymod}$ technique with Spatially Modulated Illumination Microscopy to realize a three-dimensional optical resolution of intracellular nanostructures of few tens of nm, comprising thousands of individually localized molecules[14]. Presently, these 'nanoscopy' methods are used in a variety of collaborative projects ranging from the analysis of ion channel distribution in kardiology to molecular biotechnology to radiation biophysics to the spatial analysis of telomeric structures involved in maintaining chromosomal integrity during cell division; in collaboration with the Institute of Theoretical Physics of Heidelberg University, the 'nanobiophotonic' approaches described are used to establish quantitative, predictive models of nuclear genome nanostructures to contribute to the emerging new field of "Bionuclear Physics".

From the physical principles of SPDM, even atomic optical resolution (< 1 nm, corresponding to about 1/100th of the exciting wavelength) appears to be possible. Combined with X-ray structure data of individual proteins, this might open a laseroptical avenue towards atomic resolution of the many biomolecular machines which so far have been resistant to any attempt of crystallization[1833].

Beyond molecular biophysics, SPDM is expected to have a vast application potential also in other fields of Physics, e.g. material sciences.

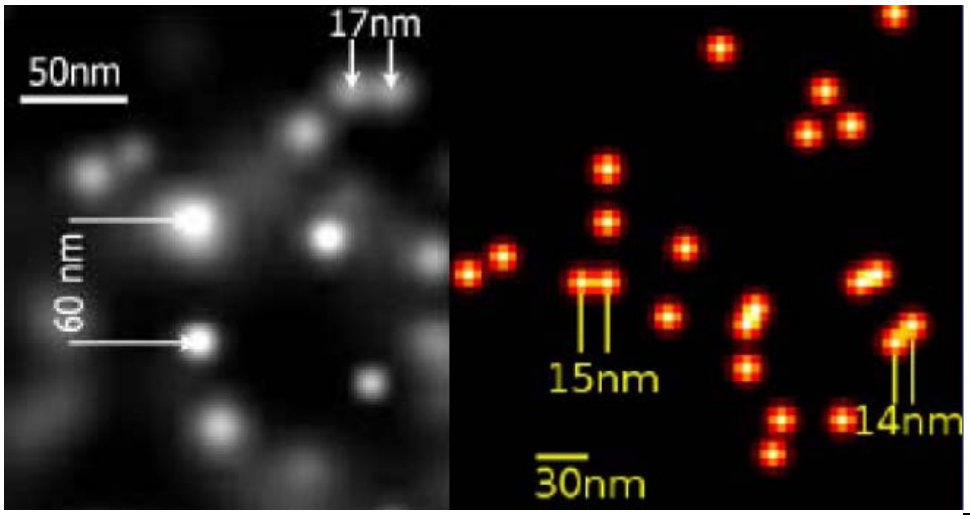

Figure 1: ***Left:*** *SPDM of individual histone (H2B) molecules in a human cell nucleus (detail)*[15]*.* ***Right:*** *SPDM of individual protein molecules in a human cell membrane (detail)*[14]*. The wavelength used for excitation of fluorescence was 488 nm.*

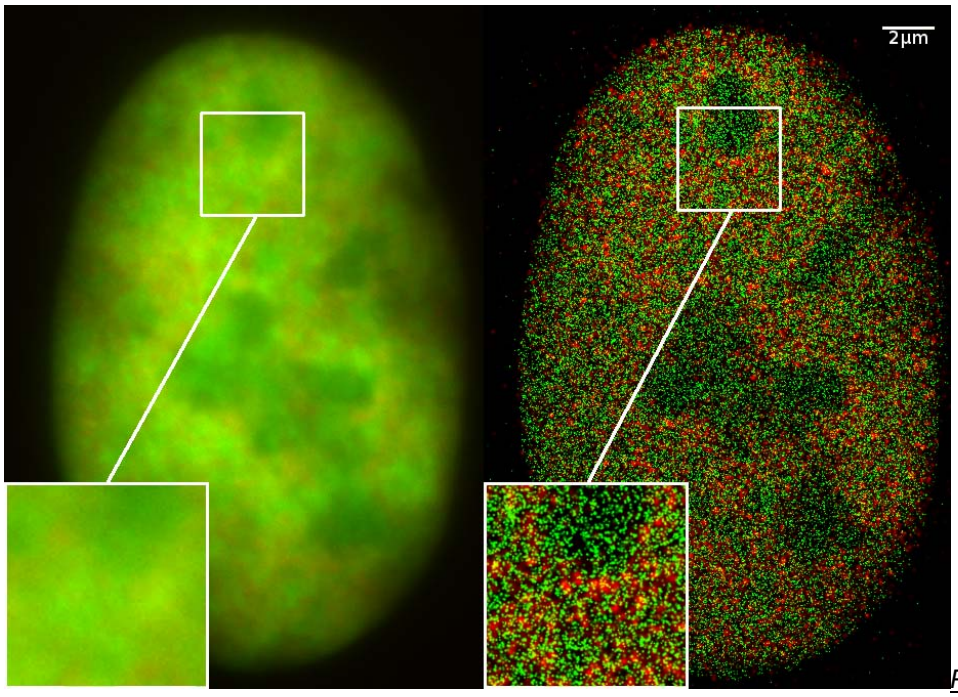

Figure 2: View of a nucleus of a bone cancer cell: ***Left:*** *Using conventional high resolution fluorescence microscopy, it is not possible to*

distinguish details of its structure. ***Right:*** *Using two Color Localization Microscopy (2CLM/SPDM) it was possible to localize 70,000 individual histone molecules (red: RFP-H2A) and 50,000 individual chromatin remodeling proteins (green: GPF-Snf2H) in a field of view of 470 µm^2 with an optical depth of 600 nm. Single molecules of the same type with a distance of few tens of nm were clearly resolved. Common fluorescence markers were used*[17].

Acknowledgments: The lightoptical nanoscopy approaches presented are the work of many members of the Cremer-Lab (see Figure 3). The support of the State of Baden-Wuerttemberg, the Deutsche Forschungsgemeinschaft (DFG), the Bundesministerium für Bildung und Forschung (BMBF), and the European Union is greatfully acknowledged.

Figure 3: Group Photo of Cremer-Lab. First row: U. Birk, D. Baddeley, G. Kreth, J. Schwarz- Finsterle, C. Wagner, H. Schneider, J. Reymann, S. Fenz; Second row: H. Eipel, C. Batram,J. von Hase, H. Mathee, C. Grossmann, C. Carl, M. Bach, S. Stein, S. Pazahanisamy, A. Schweitzer, W. Stadter, N. Kepper; (not represented in this Photo): R. Amberger, A. von Bassewitz, S. Beismanis, G. Best, A. Brunner, P. Lemmer, M. Gunkel, R. Kaufmann, T. Ruckelshausen, W. Schaufler.

Selected Publications

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2) C. Cremer & T. Cremer (1971) Method for the imaging or modification of object details with dimensions below the visible range (Verfahren zur Darstellung bzw. Modifikation von Objekt-Details, deren Abmessungen außerhalb der sichtbaren Wellenlängen liegen). German patent application No. P 21 16 521.9, filed April 5, 1971, published October 12, 1972.

*3) C. Cremer & T. Cremer (1978) Considerations on a Laser-Scanning-Microscope with high resolution and depth of field. Microsc. Acta **81**: 31-44.*

*4) S. Hell, S. Lindek, C. Cremer, E. H. K. Stelzer (1994) Measurement of the 4pi-confocal point spread function proves 75 nm axial resolution. Appl. Phys. Lett**. 64**: 1335-1337.*

*5) D. Baddeley, C. Carl, C. Cremer (2006) 4Pi microscopy deconvolution with a variable point-spread function. Appl. Opt. **45**: 7056-7064.*

6) R. Heintzmann & C.Cremer (1999) Lateral modulated excitation microscopy: Improvement of resolution by using a diffraction grating. Proc. SPIE 356, 185-196.

*7) A.V. Failla et al.(2002) Subwavelength size determination by spatially modulated illumination virtual microscopy. Applied Optics **41**: 6651-6659.*

*8) D. Baddeley et al. (2007) Nano-structure analysis using Spatially Modulated Illumination microscopy. Nature Protocols **2**: 2640 – 2646.*

9) C. Cremer et al. (1996) German patent application No. 196.54.824.1/DE, filed Dec 23, 1996; European Patent EP 1997953660, 08.04.1999, Japanese Patent JP 1998528237, 23.06.1999, United States Patent US 09331644, 25.08.1999.

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*12) M. Heilemann et al. (2002) High-Resolution colocalization of single dye molecules by fluorescence lifetime imaging microscopy. Anal. Chemistry **74**: 3511- 3517.*

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*14) P. Lemmer et al. (2008) SPDM: Light microscopy with single-molecule resolution at the nanoscale. Applied Physics B **93**: 1- 12.*

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16) R. Kaufmann et al. (2009) SPDM – Single Molecule Superresolution of Cellular Nanostructures. Proc. SPIE 7180, 71850 –J – 71850J-19.

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