# New Developments in Photonic Microscopy : Laseroptical Nanoscopy of Cellular Structures

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Organisms are made up of single cells; single cells contain a large number of highly structured macromolecule complexes ("biomolecular machines", BMMs) to perform essential tasks, such as replication, transcription and repair of DNA, RNA splicing, protein synthesis and degradation, intracellular molecular transport, or the transfer of ions and proteins across cell membranes. Typically, such BMMs have a size in order of few tens of nm to about 100 nm. Furthermore, experimental and theoretical evidence is accumulating that small chromatin domains in the cell nucleus may also form functional nanostructures and thus constitute an additional class of BMMs [1]. For example, a current topic of research is the relation between the three dimensional (3D) folding of the DNA fibers in the cell nucleus and the regulation of gene expression. Since the individual cell is the basic structure of life, it is of utmost importance for understanding the complex system of the cell to complement biochemical and molecular biology analyses (typically based on the disruption of many cells into its constituents) with the study of the individual cell itself. This requires appropriate single cell analysis tools, in particular a combination of molecular labelling and optical imaging.

While Electron microscopy and other ultrastructure imaging methods based on ionizing radiation have the great advantage of an unprecedented optical resolution, 'visible' light (range from near ultraviolet to near infrared) offers other advantages, such as simultaneous identification of multiple types of appropriately labeled molecules in single, three-dimensionally intact cells, even in living ones. Thus, it should be highly useful to complement the potential of ultrastructure procedures with novel approaches to perform high resolution analyses of cellular structures also using visible light microscopy.

Presently, light optical techniques like Fluorescence Energy Transfer (FRET) or Fluorescence Recovery After Photobleaching (FRAP)/Fluorescence Correlation Spectroscopy (FCS) allow to measure distances between two interacting molecule types down to the few nanometer level and to analyse intracellular mobilities of labelled molecules [2,3]. For a full understanding of functional cellular processes, however, additional structural information is necessary. A serious problem to achieve this goal is the conventional light microscopical resolution limited to about 200 nm laterally (about half the wavelength used) and 600 nm axially; that means that cellular nanostructures cannot be adequately resolved to provide full functional information.

Various recently introduced laser optical "nanoscopy" approaches allow to overcome this problem.

**Spectral Precision Distance Microscopy** [SPDM; 4–7]. SPDM is a method of Localization Microscopy to determine positions and mutual distances far below the conventional optical resolution limit (COR). Originally, this limit has been defined as the smallest distance detected between two point like objects (Abbe 1873); implicitly, it is assumed that both objects have the same 'color', i.e. the same spectral signature (any label by which one can 'sort' the photons registered by the two objects, characterized e.g. by differences in absorption, fluorescence spectrum, lifetime etc.). It is obvious that in the case two point like objects can be distinguished by their spectral signature and thus the diffraction images of the two objects can be registered independently from each other, the distance resolution is not any more determined by COR but by the localization precision of the individual objects and by the capability to correct for optical aberrations. In this case, in addition to the distances, one obtains also the positions of the individual objects. This idea is well established since decades in Astronomy and can also be applied to the analysis of cellular nanostructures by visible light microscopy. In this case, it comprises the following steps: 1) Molecular labelling of the nanostructures to be analysed in such a way that in a given observation volume (corresponding to the Full-Width-at-Half-Maxima [FWHM[ of the Point Spread Function [PSF] of the microscope system used), at a given time only one object/molecule of a given spectral signature is present; various 'point like' objects/molecules within the observation volume are labeled with different spectral signatures; 2) For each spectral signature, the microscopic image is registered separately; 3) For each registered object of a given spectral signature, the object/molecule position is determined from the

respective diffraction images by appropriate algorithms, for example by adaptation of a Gaussian, a PSF, or simply by calculating its bary center (fluorescence intensity 'gravity center'); 4) Optical aberrations are carefully corrected for. Especially important are the aberrations caused by the use of different spectral signatures (e.g. chromatic aberrations); 4) The corrected positions of the objects are listed and can be presented as an image like in the 'pointilistic' paintings of impressionism. Therefore, localization microscopy techniques have also been referred to as 'pointilism' (R. Heintzmann).

In this way, the measurement of positions and mutual distances of 'point-like' fluorescent cellular structures in a range far below the conventional resolution limit was achieved. For example, confocal SPDM allowed an intracellular 3D distance resolution of about 50 nm, or 1/10 of the wavelength used. Examples for the application of SPDM in nuclear genome structure research are the analysis of the BCR-ABL region [8]; or of the distribution of genes in the active and inactive X-chromosome territory [9].

**Spatially Modulated Illumination** [SMI] far field light microscopy [10-13] allows a highly improved localization precision and is capable to measure the diameter of individual fluorescent subwavelength sized small objects with high accuracy. Its principal idea is 1) To create a standing wave field by two counter propagating laser beams. This can be achieved e.g. by focusing two beams derived from the same laser source into the back focal planes of two opposing high numerical aperture objective lenses. 2) The fluorescence labelled object structures positioned in the standing wave field are excited, according to their position; the object is moved in small steps (e.g. 20 nm, or 40 nm) along the optical axis; at each axial position, an image is registered. The axial intensity profile of a fluorescent object in the 3D data set obtained is modulated by the wave field and the size/fluorochrome distribution of the object. Both theoretical considerations/simulations, and experimental calibration results using objects of known diameter revealed that under certain conditions, the axial extension of the objects (size) can be determined down to few tens of nm. In addition, the localization precision of small fluorescent objects is highly improved (down to the 1.0 nm regime). Several application examples indicated the usefulness of SMI-"nanoscopy" for the study of gene compaction on the level of nuclear protein complexes like transcription factories [14] and individual gene regions [15, 16].

**Confocal laser scanning 4Pi-microscopy** is a microscope approach to scan the object by laser light focused from all sides ("4Pi" geometry) and to detect confocally the fluorescence excited [17]. Using two opposing high numerical aperture lenses, confocal laser scanning 4Pi-microscopy [18- 20] has now become an established "nanoscopy" method, allowing an axial optical resolution and hence a size determination down to the 100 nm regime. Recently, this method has been offered by a commercial manufacturer and introduced in a number of biophysically oriented laboratories. Application examples presented are the analysis of ion channel distribution in the cell membrane (collaboration with E. Zitron, C. Karle, Cardiology Dept. Univ. Heidelberg) and the analysis of replication factories in the nucleus of individual cells (collaboration with M.-C. Cardoso, MDC Berlin).

**Stimulated Emission Depletion (STED) microscopy** was pioneered by Stefan Hell and his group and has made it possible to overcome by many times the classical "Abbe limit" of optical resolution. The principal idea of STED is to excite a diffraction limited small area (e.g. of 200 nm diameter) by a first laser beam; then the fluorescence in this area is depleted by a second 'STED' laser beam (using a slightly other wavelength) ***except*** a very narrow central area (e.g. of 20 nm diameter). Under these conditions, only this tiny center will emit fluorescence photons of a given energy (wavelength) and thus be registered. The optical resolution (smallest distance resolvable for objects of the same spectral signature) corresponds to the diameter of the tiny fluorescent center created by the STED process. The image is realized by point by point scanning; in the end the image can be reconstructed from the site dependent fluorescence intensities as in conventional laser scanning microscopy. Using STED microscopy, the protein distribution on membranes has been studied with an effective optical resolution of about 15 nm, i.e. down to the macromolecular level [21].

**Patterned excitation microscopy** [22, 23] provides another way to overcome the conventional "Abbe limit" of light microscopy. In this approach, a structured illumination pattern combines with high-spatial frequencies in a sample to produce low-frequency moiré patterns that are registered; mathematical techniques are then used to form a real image at enhanced resolution from multiple individual moiré images acquired using different illumination pattern orientations. The resolution enhancement depends on the spatial frequencies contained in the illuminating pattern. Presently, around 100 nm laterally and 200 nm axially are achieved.

**Photoactivated Localization Microscopy** (PALM/FPALM) was introduced recently by the E. Betzig and S. Hess groups [24,25]. A similar method was published by Rust et al. [26]. As in Spectral Precision Distance Microscopy (SPDM), (F)PALM is a localization microscopy method based on very precise (nanometer) positioning of subwavelength sized objects, in this case single molecules. Whereas SPDM, however, requires objects of different spectral signature to resolve them spatially within the observation volume given by the microscope used, (F)PALM allows an effective optical resolution in the 10 nm range even in the case that all objects are labelled with the same type of fluorochrome.
To achieve this, the cellular structure to be analysed is labeled with fluorescent molecules (e.g. proteins) which can be photoactivated. Photoactivation occurs stochastically and when performed in the low dose regime only a small fraction of molecules will be activated. By appropriately small intensities, just one molecule within an observation volume is activated. This enables the discrimination of single molecules which would otherwise be indistinguishable from their neighbors. These activated molecules are then excited to fluorescence emission by a read out laser beam. By determining the center of the fluorescence emission of a photoactivatable molecule through a statistical fit of an approximate PSF to its measured photon distribution, the position of the molecule can be determined with an accuracy considerably superior to the conventional optical resolution limit of ~200nm (laterally). By bleaching the fluorophores irreversibly during the fluorescence read out and repeating the activation and measurement steps, another fraction of the molecules can be measured. After an appropriate number of repetitions (e.g. 20,000) an image with a much improved (few tens of nanometers) spatial resolution can be obtained.

Instead of spatial scanning of the object like in 4Pi-, STED, SMI, or patterned excitation microscopy, this microscopy approach requires the registration of multiple (thousands) of images of the same specimen, i.e. the optical resolution is improved by 'scanning' the fourth coodinate of the space-time continuum. If the smallest size of photoswitchable fluorophores is assumed to be in the 1 – 2 nm range, then (F)PALM should in principle allow to realize an effective optical resolution at the molecular level; it should be combined advantageously with other nanoscopy approaches, e.g. SMI/patterned excitation, STED, or SPDM.

To summarize, it is anticipated that these and other novel developments in laseroptical nanoscopy will eventually bridge the gap between ultrastructure methods (nm optical resolution) and visible light far field microscopy (conventionally hundreds of nm optical resolution) in such a way that the same cellular structure can be imaged at almost similar (down to molecular) spatial resolution. This will provide an essential contribution to a direct insight into the 'machinery' of life on the individual cell level, from the replication and repair of DNA to the change in folding of the chromatin fiber at the activation/silencing of a gene, to its transcription, to the processing of the mRNA produced, to the transport of mRNA to the cytoplasm through the nuclear pores and its translation into proteins, to the assembly and disassembly of macromolecular complexes, up to the signal transduction at the cell membrane and the molecular interaction between neigboured individual cells. Beyond these exciting prospects for fundamental biophysical research, it is anticipated that laseroptical nanoscopy methods will also provide additional valuable tools for the analysis of the interaction of 'biomolecular machines' and pharmaceutical drugs on the level of single cells/single BMMs.

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