Far-field Light Microscopy

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Novel developments in optical technology and photophysics made it possible to radically overcome the diffraction limit (ca. 200 nm laterally, 600 nm along the optical axis) of conventional far-field fluorescence microscopy. Presently, three principal ‘nanoscopy’ families have been established: Nanoscopy based on highly focused laser beams; nanoscopy based on structured illumination excitation; and nanoscopy based on localisation microscopy approaches. With such ‘superresolution’ or ‘nanoscopy’ techniques, it has become possible to analyse biostructures with a substantially enhanced light optical resolution down to a few tens of nanometre in 3D, and a few nanometre in the object plane, corresponding to 1/100 of the exciting wavelength. These methods allow to study individual membrane complexes, cellular protein distributions, nuclear nanostructures, bacteria or individual viruses down to the molecular level; they open new perspectives to combine molecular and structural biology to unravel the basic mechanisms of life and their emergence from fundamental laws.

Introduction

Far-field light microscopy has reemerged as one of the major tools of modern cell biology. This is due to the combination of advanced instrumentation with molecular labelling techniques using multispectral/photoswitchable (‘blinking’) fluorophors. In this article, the author describes the approaches allowing enhanced resolution far beyond the conventional limits. Presently, an optical resolution of fluorescence labelled biostructures in the 5 nm range has been achieved, corresponding to approximately 1/100th of the exciting wavelength. Here, the focus will be the application of far-field light microscopy in the analysis of nuclear genome nanostructure.

Historical Remarks

Light microscopy is regarded as one of the most important inventions in the history of humankind: in the hands of Antony van Leeuwenhoek and Robert Hooke, in the second half of the seventeenth century, it allowed the first glimpse into the microcosm of life. In the nineteenth century, progress in instrumentation and staining techniques made possible the discovery of the cell nucleus and of the chromosomes as basic constituents of eukaryotic genomes. The following further development of light microscopy and the steady increase in optical and structural resolution constituted the basis of modern biology and medicine.

State of the Art

Conventional resolution limits imposed by the wave theory of light

Since the work of Abbe and Rayleigh at the end of the nineteenth century, wave theory appeared to impose an absolute limit on the potential of light microscopy as a tool for studying the nanostructure of thick transparent specimens such as cells and cell nuclei. In an advanced conventional epifluorescence light microscope or confocal laser scanning fluorescence microscope (CLSM), the optical resolution is limited laterally to approximately 200 nm in the object plane and to approximately 1 μm (CLSM approximately 600 nm) in the direction of the optical axis of the microscope system (in the following called conventional resolution). To define the term ‘resolution’ in a biologically meaningful context, various definitions can be used (Cremer and Masters, 2013). The most important are ‘optical resolution’ (the smallest detectable distance between two object points) and ‘structural resolution’ (the finest detectable structural detail). An enhancement in structural resolution requires an appropriate improvement in optical resolution in combination with a sufficient density of independently detected object points.
Achievements in human genome research using conventional resolution light microscopy

The conventional optical resolution achieved, especially using confocal laser scanning microscopy (CLSM) techniques, is already sufficient to study many topics relevant to human genome structure: for example, to perform genome-wide cytogenetic analysis of mitotic chromosomes and to identify chromosomal band regions down to the level of several megabase pairs (Mbp) in deoxyribonucleic acid (DNA), using multicolour fluorescence in situ hybridisation (FISH) techniques; to visualise in human cell nuclei multicolour labelled chromosome territories (Bolzer et al., 2005), chromosome arm territories and still smaller chromatin domains down to the level of about 1Mbp of DNA; to identify individual genes and estimate their spatial distribution; to localise in live cells individual DNA sequences, using for example, lac operator/repressor recognition; to visualise protein and protein/ribonucleic acid (RNA) complexes related to genome function; to measure the mobility of individual protein and RNA molecules in the nucleus of living cells using fluorescence recovery after photobleaching (FRAP) or fluorescence correlation spectroscopy (FCS) techniques.

Biological problems requiring further enhancement of resolution

Compared with the typical size of nucleosomes (11 nm diameter); of the chromatin domains of individual genes (e.g. 100 kbp corresponding to a size estimate in the order of 100 nm); or of the ‘biomolecular machines’ for replication, transcription, splicing and repair, the present resolution of the light microscope is by far insufficient to answer many pressing questions in human genome structure research, such as the extent of an interchromatin domain compartment; the relative positioning of specific genes with respect to chromatin domains; the spatial structure and temporal dynamics of specific gene regions; the spatial requirements for accessibility of specific proteins to transcription factor binding sites; the assembly and disassembly of genome-related ‘biomolecular machines’ or ‘modules’; or the analysis of small changes in the compactness of a specific regulatory gene region (Mercer et al., 2013) as a prerequisite for or as a consequence of transcription, repair or epigenetic modification (Cremer and Cremer, 2001). To meet these challenges, a variety of specific questions may be asked, for example, on the position of individual gene domains with respect to the chromosome territory subdomains they belong to; their size and genetic activity (e.g. Are condensed regions inactive and decondensed ones active?); 3D-structure changes by environmental agents (ionising/UV radiation, chemical compounds) and the repair processes induced by such agents; the role of epigenetic methylation/histone modification patterns for the 3D-structure of specific domains; how are time dependent changes of the 3D-structure (folding of the chromatin fibre) related to transcriptional activity?; the accessibility of transcription factors to individual regulatory DNA sequences; the topology of transcription factories with respect to chromatin; 3D-structure changes by modifications in regulatory sequences; 3D-structure and transcription activity changes by small RNAs, such as mi-RNA; maintenance of epigenetically/environmentally related structural changes; genome nanostructure and differentiation (e.g. from stem cells to specific cell types); genome structure and evolution; genome structure and aging; genome structure and the detection of disease related cells (e.g. metastatic tumour cells).

Resolution enhancement by alternatives to far-field light microscopy

To approach the problems indicated above by far-field optical imaging methods (i.e. the distance between the objective lens and the object is in the order of multiple wavelengths), as well as myriads of other questions in cell biology, a further substantial enhancement in the resolution of light microscopes will be necessary. For many decades, however, such further improvement in resolution appeared to be impossible because of the limits imposed by the wave theory of light. Consequently, alternative methods were developed (for review see Cremer and Masters, 2013). In particular, X-ray crystallography and electron microscopy allowed further enormous progress in the elucidation of genome ultrastructure and related protein/DNA complexes. In addition, surface-related techniques like atomic force microscopy (AFM) or near-field scanning optical microscopy (NSOM) allowed the study of reconstituted genome structures on surfaces at a resolution considerably below 100 nm. These techniques contributed widely to genome structure research. None the less, only far-field light microscopy would allow the nondestructive study of specifically labelled nanostructures in the interior of intact cells, such as the 3D architecture of the human genome and its modifications in the nuclei of morphologically conserved and even in living cells. Although concepts to surpass or to ‘break’ the Abbe/Rayleigh limit of optical resolution in far-field light microscopy have been described already in the 1960s (Toraldo di Francia, 1955; Lukosz, 1966), biologically relevant far-field light microscopy systems with enhanced resolution have been realized since the 1990s only. These approaches have been labelled by various general names, such as ‘nanoscopy’ or ‘superresolution microscopy’.

Focused nanoscopy

In the 1970s, first ideas to overcome the conventional optical resolution limit by a confocal laser scanning fluorescence microscopy approach with coherent light focused from all directions by means of a ‘4π hologram’ have been put forward (Cremer and Cremer, 1978). The basic idea behind was to scan the object ‘point-by-point’ by
a focused laser beam like in CLSM but to increase the optical aperture beyond the limits of conventional microscopy (which uses one objective lens only for imaging) and thus to narrow the width of the scanning laser beam beyond the conventional limits. Theoretical considerations indicated that a full $4\pi$-angle scanning microscope (i.e. focusing the laser beam from all directions) might allow an isotropic optical 3D resolution in the 80 nm range, assuming 488 nm laser excitation, and approximately 40 nm at 257 nm excitation. Although the idea to use a ‘$4\pi$ hologram’ to achieve a full sterical angle of $4\pi$ so far has not been realized, a confocal laser scanning ‘$4\pi$ microscope’ with a somewhat smaller aperture angle using two opposite high numerical aperture lenses has been constructed in the 1990s (Hell et al., 1994; Hänninen et al., 1995).

With such a two lens $4\pi$ microscope, an axial optical resolution of approximately 90–100 nm was achieved, corresponding to a 5–7 fold enhancement in the 3D resolution compared with a conventional CLSM. Figure 1 shows an example of the application of this instrument type.

**Figure 1** Two-colour 4Pi microscopy images of PML bodies. Immunostaining was conducted with a secondary antibody labelled with Alexa Fluor 568 (PML, red colour) and Atto 647 (green colour) against (a) a SUMO-1 or (b) a SUMO-2/3 primary antibody. 3D image reconstructions of the 4Pi stacks are also shown (right). Scale bars: 0.5 \( \mu \)m (the same in x, y object plane; and in z axial direction). The first three image columns (from left) show the PML, SUMO and corresponding merged image (merge 1) of two PML-NBs. Then the merged PML-SUMO image of two other PML-NB is presented (merge 2). SUMO-1 was distributed more sparsely and also more aggregated than PML. A partial colocalization of PML (red) and SUMO-1 (green) in the same spherical shell was evident from the line profiles 1 and 2 shown in (c). By contrast, SUMO-2/3 was located also in the interior of the PML-NB (b, merge 1). A fraction of PML-NBs showed only a very weak SUMO-2/3 signal in the interior (b, merge 2). (c) Image-intensity profiles taken along the broken lines indicated by white numbers in a (1,2) and in b (3); red: PML; green: SUMO. (d) The 3D-structure of a PML-Sp100 4Pi-reconstruction is shown for comparison. Sp100 was distributed similarly to PML in the outer shell of spherical shape. No extrusions from the shell were apparent but the two proteins were present in distinct patches. Reproduced from Lang et al. (2010). © Company of Biologists Ltd.
in the analysis of promyelocytic leukaemia nuclear bodies (PML-NBs) in human cancer cells (Lang et al., 2010). PML-NBs are mobile subnuclear organelles formed by PML and Sp100 protein PML bodies.

Although the axial optical resolution was enhanced by a factor 5–7 compared to conventional CLSM, the lateral resolution (object plane) was only slightly improved, due to the missing cone in the sterical aperture angle in this two lens 4Pi system.

**STED microscopy**

In the following years, additional approaches of scanning beam based ‘focused nanoscopy’ were developed to substantially enhance the resolution also in the object plane. The basic idea to do this was to ‘switch off’ the molecules in the vicinity of the intensity maximum of the scanning laser beam, and in this way register only the fluorescence of the molecules at the beam centre. Since the position of the scanning beam can be determined from the microscope mechanics with nanometre precision, one can localise different fluorescent molecules independently from each other even if their distance is substantially lower than 200 nm. This ‘switching off’ can be realized e.g. by a basic photophysical mechanism called stimulated emission; since by such a stimulated emission the number of registered fluorescent molecules in the vicinity of the beam centre is strongly reduced (‘depleted’), this method has been called stimulated emission depletion (STED) microscopy (Hell and Wichmann, 1994; Schrader et al., 1995; Hell, 2007). So far, using STED microscopy, an effective lateral optical resolution of approximately 10–20 nm was realised in biological specimens. This means that fluorescent molecules were distinguished from each other (resolved) which had a distance of only 10–20 nm from each other. Using solid state objects, the present limit of lateral optical resolution of STED microscopy is in the range of 5 nm (using optimum conditions), corresponding to about one-hundredth of the exciting wavelength. In theory, STED microscopy would even allow a still better resolution (Hell, 2009).

The concepts outlined above for STED microscopy were generalised for an entire class of focused nanoscopy approaches, called RESOLFT (reversible saturable optical fluorescence transitions) microscopy (Hell, 2009). This principle states that superresolution by any kind of scanning device can be obtained if illumination conditions can be realised with focal spots (or other modulated intensity distributions) featuring central intensity zeros which allow one to inhibit transiently the reversible fluorescence emission of molecules outside this zero region, for example, by stimulated emission (depletion of excited molecular energy levels), or by ground state depletion (depletion of ground state energy levels).

**Structured illumination excitation (SIE) microscopy**

Instead of scanning the object with a focused laser beam, the object may be scanned also with a pattern of exciting light, and without the necessity to inhibit transiently the reversible fluorescence emission of molecules outside the zero intensity region as in the RESOLFT concept. According to the structural information enhancement desired, various approaches have been described, such as spatially modulated illumination (SMI) and patterned excitation/structured illumination microscopy (PEM/ SIM).

**Spatially modulated illumination microscopy (SMI)**

SMI microscopy is a method of wide-field fluorescence microscopy using axially structured illumination (i.e. modulation of the illuminating light along the optical axis) to obtain additional high resolution information about sizes and relative positions of small fluorescently labelled target regions. To generate the illumination pattern, a standing wave field of light is established, in an analogous way as the standing waves created by two water waves moving against each other. In SMI microscopy, two oppositely directed, collimated laser beams are brought to interference by using two opposite objective lenses (like in 4Pi microscopy but without focusing), thus establishing a standing wave field with a periodic alternation of maxima and minima of light intensity. The object is moved through this standing wave field in precise axial (z) steps (i.e. along the axis through the two objective lenses), and at each step (e.g. Δz = 20 nm) an image is taken (Baddeley et al., 2007). The dozens of images obtained are combined to obtain improved position information (experimentally down to the subnanometer range, approximately 1/900th of the exciting wavelength), or to measure the size of small fluorescent molecule complexes down to few tens of nm. In this way, for example, the size of replication and transcription factories has been determined to be in the range of 80–100 nm, that is, far below the conventional optical resolution limit (Martin et al., 2004; Baddeley et al., 2010).

Figure 2 shows an example for the SMI ‘nanosizing’ of replication foci in a mouse cell line. In this case, an average diameter of the replication foci of about 120 nm was obtained.

SMI microscopy may also be used to measure the size of a small specific chromatin region in a cell nucleus (Hildenbrand et al., 2005): the condensation status of a gene domain is regarded to have a decisive influence on its genetic activity; thus, size measurements may provide key information towards a better understanding of the regulation of gene activity (Mercer et al., 2013).

In contrast to focused nanoscopy techniques, the SMI method as originally described for two opposing collimated laser beams having a fixed angle to each other was not suited for the generation of enhanced (two-point) optical resolution images of photostable fluorescence emitters, due to a range of missing intermediate spatial frequencies. This goal was realized by using patterned/structured illumination providing an excitation intensity modulation in the object plane.
Patterned excitation/structured illumination/ patterned excitation microscopy (PEM/SIM)

Experimental approaches to overcome the conventional optical resolution limit of far-field fluorescence microscopy in the lateral direction (object plane) by a single objective lens approach using laterally structured/patterned illumination were realized by the end of 1990s (Heintzmann and Cremer, 1999; Gustafsson, 2000; Heintzmann and Gustafsson, 2009) and have presently found a variety of biomedical applications, e.g. in the study of the nuclear envelope (Schermelleh et al., 2008). The basic principle of these approaches was to create a SMI pattern ('standing wave field') not in the direction (z) along the optical axis as in SMI microscopy but in the object plane (x,y). This can be done, e.g., by inserting a diffraction grating in the illumination beam at the conjugate object plane and projecting it through the objective lens into the object. Instead of a diffraction grid which is projected into the object plane, one may create the desired illumination pattern (including a modulation along the optical axis) also by the interference of two or more laser beams (Best et al., 2011). The object and the illumination pattern are then moved relative to each other in precise (x,y,z) steps. At each step an image of the entire field of view is taken, e.g., by a CCD camera (in contrast to focused nanoscopy approaches where the fluorescence is excited and detected point-by-point). The ‘wide-field’ images obtained are used to calculate an image with enhanced resolution using an algorithm based on the structure of the Fourier space. Principally, the effective optical resolution (i.e. the resolution of the calculated image) can be improved up to a factor of 2 compared with conventional wide-field microscopy. Typical resolution values achieved by SIM/PEM are about 100 nm laterally (object plane) and 350 nm along the optical axis. Figure 3 gives an example for a SIM application in nuclear structure research.

Spectrally assigned localisation microscopy (SALM)

Localisation by light has always played a fundamental role in science. In many cases, the information one wants to obtain by microscopic analysis is the localisation and the mutual distances (topology) between specific structural elements, for example, specific fluorescent molecules or small complexes of molecules. This is also the case in nuclear genome structure research where genome topology in three-dimensionally intact cell nuclei is under investigation (Cremer and Cremer, 2001). Each of the fluorescent molecules registered in a far-field microscope produces a diffraction pattern in the image plane; two neighbouring molecules can be optically resolved only if the intensity maxima of the diffraction patterns can be distinguished from each other. If both molecules are simultaneously emitting light of constant intensity at the same wavelength, the minimum detectable distance is given by the conventional resolution limit of ca. 200 nm. However, a resolution enhancement far beyond these optical resolution limits is possible if the objects are labelled with fluorescent markers having different ‘spectral signatures’, for example, different absorption wavelengths, different emission wavelengths, different fluorescence life times, different luminescence behaviour etc. This allows an independent registration (‘optical isolation’) of the diffraction patterns (Airy discs) obtained from the ‘point-like’ object elements (for review see Cremer and Masters, 2013). From the centroids (fluorescence intensity gravity centres) of the diffraction patterns or from the maxima of adaptation functions to the individual diffraction patterns, the positions of the object elements (molecules) can be determined with a localisation error many times smaller than the size of the Airy discs. Hence, using the parameters of the optical system used, the positions of the ‘point-like’ object elements can be
determined with nanometre accuracy. Each object position determined is assigned to a joint localisation map. From the object positions in this localisation map, the mutual distances in the object space can then be determined (Figure 4). A first application in nuclear genome nanosctructure elucidation was based on photostable excitation/emission spectra as spectral signatures (Esa et al., 2000).

To enhance the number of usable spectral signatures, emission differences in the time domain have been used, such as fluorescence lifetimes (Heilemann et al., 2002) or photoswitching schemes (Lidke et al., 2005; Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006; Bock et al., 2007; Lemmer et al., 2008; Heilemann, 2008; for review see Cremer and Masters, 2013). In the photoswitching approach, adjacent molecules are excited in such a way that they start to ‘blink’ in a way similar to the fire of a light house, or to the position lights of an airplane, or the blinking lights on a Christmas tree: The molecule diffraction patterns are registered one after the other at different times; hence the positions of molecules can be determined (resolved) independently from each other even if their distance is substantially smaller than the conventional resolution limit. According to the conceptual, methodological and technical variations, these localisation based approaches to resolution enhancement in far-field light microscopy have been denominated with an ever increasing multiplicity of names and abbreviations, such as PALM, FPALM, SPDM, STORM, dSTORM etc. (Cremer and Masters, 2013). In

Figure 3 Structured Illumination Microscopy (SIM) of nuclear topography of nascent RNA, nascent DNA, and Ser-2P-RNA Pol II. Comparison between optical sections of a small part of a murine C127 cell nucleus obtained from deconvolved conventional wide-field images (a,b) and 3D-SIM images (a*,b*) of the same regions obtained with a commercial SIM system (OMX, Applied Precision Instruments). Ser-2P RNA Pol II (red) and Pol 3/3 (green) primary antibodies were used to mark the CTD domain repeats phosphorylated at serine 2, and the RPB1 domain of the enzyme, respectively, together with secondary antibodies conjugated to Alexa 594 (red) and Alexa 488 (green). DNA was counterstained with DAPI (grey). Scale bar, 500 nm. Reproduced with permission from Markaki et al. (2010). © Cold Spring Harbor Laboratory Press.
case the point sources to be resolved are single molecules, an
often used general name is Single Molecule Localisation
Microscopy (SMLM). All these different localisation based
methods may also simply be referred to as ‘localisation
microscopy’. Since the abbreviation LM of localisation
microscopy is ambiguous (LM also stands for light micro-
scopy in general), and since localisation microscopy as a
general principle of resolution enhancement is applicable
not only to individual molecules but to all kinds of point
sources, the abbreviation SALM mentioned above has also
been suggested (Cremer and Masters, 2013).
Presently, SALM has emerged as one of the most
valuable approaches to achieve superresolved images of
fluorescent labelled biostructures. For example, recently it
has become possible to resolve the molecular arrangement
in individual nucleopore complexes (Loschberger et al.,
2012). Another example for applications in genome struc-
ture analysis is presented in Figure 5. Here, a special variant
of SALM was used, Spectral Precision Distance/Position
Determination Microscopy (SPDM) with physically mod-
ifiable fluorophors. This special SALM-SPDM method
allowed to use various kinds of standard green fluorescent
proteins/conventional organic dyes in combination with
standard specimen preparation and even monochromatic
excitation for a given molecule type (Reymann et al., 2008;
Lemmer et al., 2008).

Figure 4 Principle of resolution enhancement by spectrally assigned localisation microscopy (SALM). Three point-like objects are assumed to be located in
the xy-plane within mutual distances of 50 nm, i.e. substantially smaller than the conventional resolution. Furthermore, they are assumed to be labelled with
the same spectral signature in (a) producing the diffraction pattern shown in (b) or with three different, unique spectral signatures B,G,R in (d), producing
the diffraction patterns shown in (e and f). In (f), the different spectral components are imaged simultaneously whereas in (e) the same signals (B,G,R) are
registered independently from each other, producing three independent diffraction patterns B,G,R. Linescans through the diffraction patterns in (b) and in
(e) B,G,R are shown in (c) and (g) respectively. Reproduced from Kaufmann et al. (2009). © SPIE.
So far, localisation microscopy systems have been constructed providing 5 different excitation laser frequencies, hence allowing the SALM analysis of up to 5 different fluorophore types simultaneously (Rossberger et al., 2013). The present optical resolution limit (smallest detectable distance between two fluorescent point sources) is about 5 nm in the analysis of flat biostructures and a few tens of nm in nuclear structure applications; 5 nm
corresponds to about 1/100th of the exciting wavelength. In the material sciences, using conventional fluorophore molecules, optical (two-point) resolution values as small as 1.3 nm have been reported, corresponding to about 1/500th of the exciting wavelength (Pertsinidis et al., 2010). This indicates that from the side of Physics, a comparable optical resolution should become possible also in the far-field fluorescence microscopy analysis of biostuctures. While the few nm resolution scale appears to be not possible to approach by SIE alone, SIE techniques should be highly useful towards this goal in a combination with localisation microscopy (Rossberger et al., 2013). ‘Virtual microscopy’ calculations as well as experimental evidence (Albrecht et al., 2002; Baddeley et al., 2007) indicate that under low photon yield conditions, SIE allows a substantial enhancement of localisation accuracy; hence a 3D localisation accuracy in the 1 nm range and hence an optical resolution in the range of 10 nm and even better should eventually become possible. In STED microscopy, a similar resolution range has been obtained in the object plane in the analysis of colour centres in a material sciences application (Rittweger et al., 2009). To what extent these achievements can be extended to the few nm scale in 3D in cells and cell nuclei has to be seen.

**Perspectives**

In combination with methods of molecular biology, fluorescence labelling techniques, and quantitative image evaluation software, far-field light microscopy will play an ever growing role in the life sciences. In the conventional resolution range, fluorescence light sheet microscopy may be cited as an example for novel basic achievements providing the possibility to study the 3D development of entire small organisms ‘in vivo’ down to the single cell level (Keller et al., 2008). For the analysis of cellular and viral nanostuctures (Muranyi et al., 2013), focused, structured, and localisation microscopy approaches are expected to be further developed, both by scientific laboratories and by commercial manufacturers; eventually, they will provide a ‘nanoscopy’ tool set to meet the various experimental challenges.

The developments in superresolution far-field light microscopy described here presently allow an optical resolution and a size resolution down to the scale of a few tens of nanometres in 3D intact cells. It is anticipated that in the future, focused, localised and structured excitation illumination systems will become available which will allow the superresolved imaging of ten or even more different fluorophores (and hence molecule types) simultaneously. For the different techniques, the selection of the appropriate fluorophores is of paramount importance. For example, while in 4Pi and structured/patterned illumination microscopy, in principle all fluorophores may be used which are also suitable for conventional fluorescence and CLSM microscopy, STED- and RESOLFT approaches may require the use of very special dyes, especially in multicolour measurements. Similar restrictions were believed to be valid also in localisation microscopy approaches: While localisation microscopy with photostable fluorophores was shown to be possible with standard dyes (Esa et al., 2000), in the beginning of photo switching based localisation microscopy specially designed photactivatable green fluorescent proteins, or special pairs of molecules were applied (Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006). In the following years, however, it turned out that many standard fluorophores can be induced to ‘blink’ under appropriate illumination and preparation condition. The ‘spectral signatures’ required for optical isolation will comprise not only the steadily increasing variety of fluorophors useful for photoswitching based localisation microscopy (Dempsey et al., 2011) but in addition (or even as an alternative) a multitude of reporter molecules/point sources with photostable excitation/emission spectra (van Oijen et al., 1998; Bornfleth et al., 1998; Cremer et al., 1999; Esa et al., 2000). The advent of extremely fast positional sensitive wide-field single-photon counting detector systems to measure
Figure 6  Virtual multicolour ‘nanoscopy’ of nuclear genome structure. (a) 3D visualisation and projections to the lateral ($x$, $y$) plane and the vertical ($x$, $z$) and ($y$, $z$) planes of a computer simulation of a 1 Mbp chromatin domain of total diameter 500 nm (Cremer and Cremer, 2001) assumed to comprise 10 condensed 100 kbp domains formed by nucleosome chains, having an individual size in the 100 nm range. The small ‘beads’ represent the individual nucleosomes. Here, a labelling of all 10 domains with the same spectral signature was assumed. (b–d) Virtual microscopy representations (3D visualisation, projections) of the monospectral computer simulated 1Mbp domain shown in (a). For this, the simulated 3D data set of (a) was convoluted with the effective point spread function for the optical resolution assumed. (b) Virtual microscopy assuming a theoretical 3D resolution of 25 nm. The positions and sizes of the ten 100 kbp regions are all resolved individually. (c) Virtual microscopy assuming an effective optical resolution of 100 nm. Some structural resolution of the subdomains is still maintained. (d) Virtual microscopy assuming an effective optical 3D resolution of 250 nm (achieved, for example, by merging confocal images obtained from different angles. All structural information has been lost, except the total size of the 1Mbp domain. (e) Virtual microscopy of (a) assuming a multicolour 3D resolution of 10 nm, realised, for example, by combinatorial localisation microscopy with four detectable differences in spectral excitation/emission. The bar indicates 100 nm. (Figure kindly provided by Dr G. Kreth, Heidelberg). (f) Multicolour localisation microscopy of FISH labelled sequence specific oligonucleotide probes would allow to unravel the folding of an individual 100 kbp subdomain. Modified from P. Diesinger, Ph.D. Thesis Physics, University of Heidelberg 2009). © P. Diesinger Scale bar 30 nm.
fluorescence lifetimes in the time domain at high temporal resolution (Hartig et al., 2014) will substantially enlarge the possibilities (Heilemann et al., 2002) to use a multitude of fluorescence life times of such photostable emitters for localisation microscopy, according to its initial general concepts (for review see Cremer and Masters, 2013). Since such ‘non-photoswitching’ types of localisation microscopy are compatible with extremely low illumination intensities and fast imaging, it should become especially suitable for the in vivo nanoscopy of biostuctures, down to the few nanometre optical resolution.

Figure 6 provides an idea of the potential of a multicolour 3D ‘nanoscopy’ of nuclear genome structure. As an example, a simulated 1 Mbp chromatin domain of 500 nm diameter with 10 subdomains (gene domains) of 100 kbp each is shown in (a). Furthermore, in (a) – (d) a monocolour labelling of the DNA sequences in the 1 Mbp domain is assumed, as obtained by FISH with appropriate domain specific DNA probes. While conventional resolution (250 nm) would not allow to detect any substructure (d), a 3D resolution of 100 nm shown in (c) obtained e.g. by a combination of structured illumination and 4Pi microscopy would at least give an information about the presence of smaller subdomains. A 3D resolution of 25 nm (b) as might be realized by an advanced STED approach (Schmidt et al., 2008) would allow to distinguish clearly between the different subdomains and even determine their individual sizes. To be able to identify all 10 subdomains (and hence the genes correlated to the nanostructural features), one would need multicolour FISH. In the combinatorial mode, 4 different colours would be sufficient (Bolzer et al., 2005). While for STED/RESOLFT microscopy, such a multicolour nanoscopy still appears to be impractical, a multicolour localisation microscopy with a similar resolution of about 25 nm in 3D (or even better) appears to be feasible (Huang et al., 2008). In this case (e), one would even be able to identify e.g. nanostructural changes in specific gene domains induced by transcription, replication, ionising and ultraviolet radiation, chemical compounds, or repair on the single cell level. This would have substantial consequences for a better understanding of these important cellular processes; it might be used for a better characterisation and identification of individual stem cells during development, or of specific cancer stem cells in a tissue section. Eventually, it might even become possible to FISH label an individual gene domain with a number of site specific oligonucleotides (Beliveau et al., 2012) with appropriate fluorphors useful for localisation microscopy. In combination with a multicolour localisation microscopy method providing a 3D resolution around 10 nm, this might allow to unravel the detailed folding pattern of such an individual gene domain (f).

Although from the point of superresolution microscopy and of multicolour oligonucleotide labelling, such a progress towards the direct imaging of the folding of specific chromatin domains appears to be feasible, it will remain a major challenge, due to the very substantial technical difficulties of such a project. In this context, a major problem will be to combine sequence specific labelling with the preservation of the 3D chromatin structure on the nanoscale. However, if successful it might change our knowledge of the molecular mechanics of epigenetic gene regulation.

For a number of basic problems in human genome research, the progress in superresolution far-field microscopy will eventually allow us to reach the resolution domain so far restricted to electron microscopy; in terms of the number of different target types simultaneously observable, facility of preparation and speed of operation, it is expected to even surpass these methods. In addition, it will make possible an old dream of the biosciences in general and of human genome research in particular: To visualise live cells on the nanoscale. At the same time, however, all the established advantages of far-field light microscopy can be maintained, namely nondestructiveness, multispectral imaging, observations of the interior of 3D-conserved (‘intact’) and eventually even living cells: Depending on the labelling method used, all nanoscopy approaches mentioned have also been shown to be compatible with live cell observations, at least to a certain degree. Since now many major elements of life (such as the genome sequence, biochemistry, individual protein sequences and their structure) are known down to the molecular level, the quantitative analysis of the cellular, and in particular the nuclear, nanostructure and its dynamics will become one of the major issues in the understanding of the specific organisation of cells and their differences, for example, in different stages of development, in different tissues or in different pathological conditions. Such an improved understanding of the ‘four dimensional’ geometry of life realized by the merging of molecular biology and novel far-field microscopy approaches will be of utmost importance for a better understanding of the structural basis of genome reprogramming, one of the fundamental problems of modern biology and medicine. See also: Chromatin Structure and Human Genome Evolution; History of the Optical Microscope in Cell Biology and Medicine

Acknowledgements

This work was supported by the Institute of Molecular Biology (IMB), Mainz; Heidelberg University; and numerous funding institutions, in particular the Deutsche Forschungsgemeinschaft (DFG), the Bundesministerium für Forschung und Bildung (BMBF); and the European Union.

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**Further Reading**


